IDENTIFICATION OF PAR_1-G PROTEIN SIGNALLING PATHWAYS INVOLVED IN THROMBIN-INDUCED CCL2/MCP-1 PRODUCTION

By Xiaoling Deng

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Centre for Respiratory Research
Rayne Institute, University College London, 5 University Street, London WC1E 6JJ (United Kingdom)

ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

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I confirm that the work contained in this thesis is entirely my own.

ABSTRACT

Uncontrolled activation of the coagulation cascade following lung injury has been implicated in both lung inflammation and fibrosis. In addition to its role in coagulation, thrombin exerts pluripotent cellular effects via the activation of its high-affinity receptor, proteinase activated receptor-1 (PAR_1). PAR_1 is a seven transmembrane domain G protein-coupled receptor that exhibits the ability to couple to multiple G protein family subunits, including $G\alpha_{i/o}$, $G\alpha_q$ and $G\alpha_{12/13}$ within the same cell type. Activation of PAR_1 on fibroblasts, a key effector cell in lung fibrosis, results in the induction of several mediators, including the potent monocyte and fibrocyte chemoattractant CCL2.

In this thesis, the G-protein and downstream signalling pathways involved in PAR_1-mediated CCL2 production and release were examined. Using a novel PAR_1 antagonist which blocks the interaction between PAR_1 and $G\alpha_q$, this thesis shows for the first time that PAR_1 coupling to $G\alpha_q$ is essential for thrombin (10nM)-induced CCL2 gene expression and protein release in murine lung fibroblasts (MLFs). The work presented here further demonstrates that these effects are mediated via the cooperation between ERK1/2 and Rho kinase signalling pathways: a calcium-independent PKC, c-Raf and ERK1/2 pathway was found to mediate PAR_1-induced CCL2 gene transcription; whereas PLC, calcium, calcium-dependent PKC and Rho kinase pathway influences CCL2 protein release. This thesis represents the first demonstration of the cooperation between two pathways in mediating the stimulatory effects of thrombin, or indeed any other extracellular stimulus, on the induction and release of the potent chemoattractant, CCL2. This thesis also examined the signalling receptor and downstream effectors involved in thrombin-induced CCL2 production in primary human lung fibroblasts (pHALFs). The results demonstrate that PAR_1 coupling to $G\alpha_q$ is also both necessary and sufficient in mediating thrombin-induced CCL2 production at low concentration of the proteinase, whereas at high concentrations, these effects may be partially PAR-independent. This discrepancy between MLFs and pHALFs may be explained by differences of PAR receptor expression between species.

Taken together, this thesis proposes that targeting the interaction between PAR_1 and specific G proteins may allow more selective blockade of PAR_1 pro-inflammatory and pro-fibrotic signalling, whilst preserving the essential role of other PAR_1-mediated cellular responses.

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Act-D	Actinomycin-D
AEC	Airway Epithelial Cells
AIP	Acute Interstitial Pneumonia
AKAP	A-kinase Anchor Proteins
ALI	Acute Lung Injury
APC	Activated Protein C
APS	Ammonium Persulphate
ARDS	Acute Respiratory Distress Syndrome
α-SMA	α-smooth Muscle Actin
ATP	Adenosine Triphosphate
BALF	Bronchoalveolar Lavage Fluid
BCA	Bicinchoninic Acid
bFGF	Basic Fibroblast Growth Factor
BOOP	Organizing Pneumonia
bp	Base Pair
BSA	Bovine Serum Albumin
cAMP	cyclic Adenosine Monophosphate
Ca^{2+}	Calcium Ions
CCL	CC Chemokine Ligand
CCR	CC Chemokine Receptor
cDNA	Complementary DNA
CFA	Cryptogenic Fibrosing Alveolitis
CHX	Cycloheximide
COP	Cryptogenic Organising Pneumonia
COX-2	Cyclooxygenase-2
CTGF	Connective Tissue Growth Factor
DAG	Diacylaglycerol
DIP	Desquamative Interstitial Pneumonia
DMEM	High-glucose Dulbecco's Modified Eagle's Medium
DNA	Deoxyribonucleic Acid
DTT	Dithiothreitol
ECL	Enhanced Chemiluminescence
ECM	Extracellular Matrix
EDTA	Ethylenediaminetetracetic Acid

EGFP	Enhanced Green Fluorescent Protein
ELISA	Enzyme-linked Immunoassay
EMT	Epithelial-mesenchymal Transition
Eo	Eosinophil
Ep	Epithelial cells
EPCR	Endothelial Protein C Receptor
ERK	Extracellular Signal Related Protein Kinase
ET-1	Endothelin-1
FCS	Foetal Calf Serum
FGF2	Fibroblast Growth Factor 2
FITC	Fluorescein Isothiocyanate
FSP-1	Fibroblast Specific Protein-1
FXa	Coagulation Factor X (active)
GAP	GTPase-activating Proteins
GDIs	Guanine-nucleotide Dissociation Inhibitors
GEFs	Guanine-nucleotide Exchange Factors
GRKs	G protein Receptor Kinases
GPCR	G-protein-coupled Receptors
HABP	High Affinity Receptor Binding Peptide Probe
HEKs	Human Embryonic Kidney Cells
HFL-1	Human Feotal Lung Fibrobast-1
HRP	Horseradish Peroxidase
HUVECs	Human Vascular Endothelial Cells
ICAM-1	Intracellular Adhesion Molecule-1
IFN-γ	Interferon-gamma
IGF-1	Insulin-Like Growth Factor-1
IIP	Idiopathic Interstitial Pneumonia
IL	Interleukin
ILD	Interstitial Lung Disease
IP3	Inositol Triphosphate
IPF	Idiopathic Pulmonary Fibrosis
JNK	Jun N-terminal kinase
kDa	Kilodalton
KO	Knockout
LARG	Leukaemia-associated Rho guanine-nucleotide exchange factor
LIP	Lymphoid Interstitial Pneumonia

LPS	Lipopolysaccharide
Ma	Mast cells
Mac	Macrophage
MAP kinase	Mitogen-activated Protein Kinase
MCPs	Monocyte Chemotactic Proteins
MEFs	Mouse Embryonic Fibroblasts
MIP-1α	Macrophage-Inflammatory Protein-1α
MKPs	MAP kinase Phosphatases
MLC	Myosin Light Chain
MLFs	Mouse Lung Fibroblasts
MMPs	Matirx Metalloproteinases
mRNA	Messenger Ribonucleic Acid
Myo	Myofibroblasts
Ne	Neutrophil
NSIP	Nonspecific Interstitial Pneumonia
PARs	Proteinase-activated Receptors
PBS	Phosphate-Buffered Saline
PDGF	Platelet-derived Growth Factor
PGE2	Prostaglandin E2
pHALF	primary Human Adult Lung Fibroblast
PIP2	Phophoinositide Hydrolysis
PKA	Protein Kinase A
PKC	Protein Kinase C
PLC-β	Phospholipase-β
PTX	Pertussis Toxin
PVDF	Polyvinylidene Difluoride
RANTES	Regulated on Activation Normally T-cell Expressed and Secreted
RNA	Ribonucleic Acid
ROCK	Rho Kinase
RT-PCR	Reverse Transcription-polymerase Chain Reaction
SDS	Sodium Dodecyl Sulphate
SP-C	Surfactant Protein C
Sphk-1	Sphingosine kinase-1
SRC	pp60src and related kinases
SRF	Serum Response Factor

SSc	Systemic Sclerosis
TF	Tissue Factor
TFPI	Tissue Factor Proteinase Inhibitor
TGF-α	Transforming Growth Factor-α
TGF-β	Transforming Growth Factor-β
THBS-1	Thrombospondin-1
TIMPs	Tissue inhibitors of metalloproteinases
TNF-α	Necrosis Factor-α
UIP	Usual Interstitial Pneumonia
uPA	Urokinase-type Plasminogen Activator
VCAM-1	Vascular Adhesion Molecule-1

Chapter 1: Introduction

1.1 Pulmonary fibrosis

Pulmonary fibrosis is a pathological process that is classically characterized by abnormal tissue repair in response to acute or chronic lung injury, resulting in the replacement of normal functional tissue with excessive and disorganised extracellular matrix (ECM) proteins, such as collagen in the pulmonary interstitium (Figure 1.1). The pulmonary interstitium is defined as the alveolar walls (including the epithelium and capillary endothelium), septae and the perivascular, perilymphatic and peribronchiolar connective tissue. Pulmonary fibrosis is a progressive illness that ultimately leads to the loss of lung function and severe respiratory insufficiency, and is an important cause of morbidity and mortality in patients.

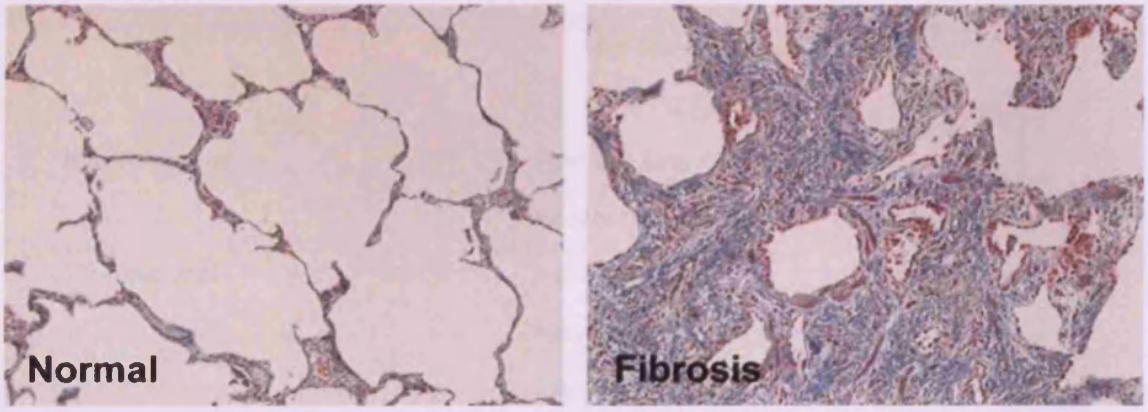


Figure 1.1 Alveolar architecture of normal and fibrotic lung. Images show the stark contrast in alveolar architecture in the normal and fibrotic lung. In the fibrotic lung, the open alveolar architecture is obliterated and replaced with dense fibrotic tissue. (Images taken from (Chambers, 2008))

Pulmonary fibrosis is a common consequence and often a central feature of many interstitial lung diseases (ILDs). ILDs are a heterogeneous group of over 200 different disorders that may share a similar clinical and radiographic presentation, but have distinct aetiologies and pathophysiology. A range of varied conditions give rise to ILDs. These include diseases with a well defined aetiology (i.e. radiation, occupational exposure to substances such as asbestosis, drug-induced lung disease), or diseases which progressively affect the lungs as one part of systemic involvement such as granulomatous diseases (i.e. sarcoidosis), and connective tissue diseases (i.e. rheumatoid arthritis, scleroderma). The ILDs with known causes have been grouped together as shown in table 1.1.

Aetiology	Clinical example
Systemic diseases	
Collagen vascular diseases	Scleroderma
	Rheumatoid arthritis
	Sjörgen's syndrome
	Polymyositis/dermatomyositis
Immunodeficiency	Common variable immunodeficiency
Exposures	
Vocational	Hypersensitivity pneumonitis
	Hot tub lung
	Bird breeder's lung
	Compost lung
Environmental	Farmer's lung
	Environmental moulds
Occupational	Asbestosis
	Berylliosis
	Silicosis
	Popcorn worker's lung
Drug-induced lung disease	Amiodarone
	Methotrexate
	Nitofurantoin
	Bleomycin
Genetic	Familial interstitial pneumonia
	Hermanski-Pudlack syndrome
	Surfactant protein-C mutation

Table 1.1. Clinical classification of ILDs (adapted from (Laurent and Shapiro, 2007))

However, most cases of ILDs have no known aetiology and are termed as idiopathic interstitial pneumonias (IIPs). In the past, the classification of IIPs has always been a problem as many of IIPs closely mimic each other clinically but the outcome of each can vary greatly. In 2002, the American Thoracic Society (ATS) and European Respiratory Society (ERS) consensus panel reclassified IIPs and concluded that IIPs comprised a number of clinicpathological entities which were sufficiently different from one another to be designated as separate diseases. As shown in table 1.2, IIPs are classified into seven distinct disease entities which can be distinguished on the base of clinical, radiological and histological characteristics.

Histological pattern	**Diagnosis based on clinical, radiological, and histological findings**
Usual interstitial pneumonia (UIP)	Idiopathic pulmonary fibrosis (IPF)
Nonspecific interstitial pneumonia (NSIP)	Nonspecific interstitial pneumonia (NSIP)
Organizing pneumonia	Cryptogenic organizing pneumonia (COP)
Diffuse alveolar damage	Acute interstitial pneumonia (AIP)
Respiratory bronchiolitis	Respiratory bronchiolitis-associated interstitial lung disease (RBILD)
Desquamative interstitial pneumonia	Desquamative interstitial pneumonia (DIP)
Lymphoid interstitial pneumonia	Lymphoid interstitial pneumonia (LIP)

Table 1.2 Classification of the Idiopathic Interstitial Pneumonias (Table adapted from: American Thoracic Society/European Respiratory Society International Multidisciplinary Consensus Classification of the Idiopathic Interstitial Pneumonias, 2002)

Of all IIPs, UIP has the poorest prognosis, whereas NSIP has a better outcome. UIP is the histopathologic pattern that identifies patients with Idiopathic pulmonary fibrosis (IPF) in lung biopsy specimens. IPF, also known as cryptogenic fibrosing alveolitis (CFA), is the most common and fatal of the idiopathic interstitial pneumonias. The typical lung biopsy of IPF is seen with areas of normal appearing lung tissue interspersed with dense scarring, honeycombing, fibrotic foci and inflammatory cells. Honeycombing is due to enlarged and distorted airspaces resulting from destruction of normal lung architecture; fibrotic foci are areas of proliferating fibroblasts with

connective tissue. Although the aetiology of IPF remains unknown, a number of environmental factors such as smoking (Baumgartner *et al.*, 1997) and viral infection (Tang *et al.*, 2003) have been implicated. Genetic polymorphisms may also account for the prevalence of IPF as the existence of polymorphisms in surfactant protein C mutation (SP-C) (Lawson *et al.*, 2004), interleukin-1 (IL-1) receptor antagonist (Whyte *et al.*, 2000), as well as polymorphism of the tumour necrosis factor-alpha (TNF-α) (Whyte *et al.*, 2000) gene and complement receptor 1 gene (Zorzetto *et al.*, 2003) found in familial cases of IPF. However, most cases currently have no known risk factor. Clinically, the most common symptoms of IPF are dysponea, poor exercise tolerance and a non-productive cough, as well as systemic symptoms such as fever and weight loss.

Epidemiological data on pulmonary fibrosis is scant and varies in the literature, but it is known that IPF is the most common cases of IIPs, accounting for more than 45% of all cases (American Thoracic Society (2000)). There is no geographical pattern of disease distribution and no racial or ethnic predisposition. In a large epidemiological study in 1994, the prevalence of all interstitial lung diseases was reported to be 80/100,00 of the general population for men and 60/100,00 for women. Of these cases, the prevalence of IPF was found to be 7-20 individuals per 100,000 population (Coultas *et al.*, 1994). However, a recent estimate has suggested a prevalence in the United States ranging from 14.0 to 42.7 cases per 100,000 population (Raghu, 2006), which is higher than that previously reported. It is further estimated that the incidence of IPF in the United Kingdom has dramatically increased by 11% between 1991 and 2003, and the overall incidence rate per 100,000 person-years is 4.6, which has more than doubled (Gribbin *et al.*, 2006). This disease occurs predominantly in men. Most patients with IPF are diagnosed between the ages of 40 and 70 and the incidence increases with age. The survival rate of pulmonary fibrosis remains significantly low with a median survival rate between 3 and 5 years (Hubbard *et al.*, 1998; Mapel *et al.*, 1998).

Despite improvements have been made in our understanding of the biochemical and biological mechanisms of pulmonary fibrosis, this lung disorder continues to pose major clinical challenges since effective therapies have yet to be identified and developed. Current therapeutic options include conventional anti-inflammatory therapy such as corticosteroids, immunosuppressants such as azathioprine, and anti-fibrotic treatment such as pirfenidone. Unfortunately, anti-inflammatory treatment with corticosteroids is associated with side effects and significant morbidity (Collard and King, 2001; Flaherty

et al., 2001a). The anti-fibrotic agent pirfenidone (Azuma *et al.*, 2005) and the anti-oxidant agent acetylcysteine showed early promise in recent IPF trials but future long-term studies are required to clarify their safety and efficacy (Demedts *et al.*, 2005).

In summary, pulmonary fibrosis such as IPF represents a severe clinical problem, and a better understanding of the pathogenesis underlying disease progression will be pivotal for the development of urgently needed effective therapies.

1.2 Pathogenesis of pulmonary fibrosis

Although during the past decades significant advances have been made in the understanding of the pathogenesis of pulmonary fibrosis, the exact mechanisms underlying the development of pulmonary fibrosis are still not fully understood. In particular, the concepts of pathogenesis of pulmonary fibrosis have changed within past decades. Initially, it was believed that pulmonary fibrosis is the consequence of chronic lung inflammation. The second hypothesis suggests that pulmonary fibrosis results from epithelial cell injury and abnormal wound repair in the absence of preceding inflammation (Strieter, 2002). The third hypothesis is based on the previous two hypotheses and postulates that injury to the lung causes subsequent inflammation, whereas the body's immune response to the injury results in pulmonary fibrosis (Strieter, 2005). A very recent review hypothesized that IPF develops as a consequence of abnormalities resulted from multiple biological pathways that affect inflammation and would repair (Maher *et al.*, 2007).

Recent progress in our understanding of the pathogenesis of pulmonary fibrosis mainly comes from studies on animal models of lung fibrosis, the commonest of which is bleomycin-induced pulmonary fibrosis model. Bleomycin can induce lung injury, inflammation and fibrosis, with many features similar to those seen in humans with pulmonary fibrosis. Both human and murine model of pulmonary fibrosis are characterized by alveolar epithelial cell injury which occurs early in the pathogenesis (Plataki *et al.*, 2005). Alveolar and airway epithelial cells (AEC) are primary target cells of lung injury. Following lung injury, the more susceptible type I epithelial cells die and detach from alveolar walls, whilst the type II cells may then proliferate and repopulate the denuded basement membrane (Haschek and Witschi, 1979). In pulmonary fibrosis, the alveolar epithelium is thought to fail to regenerate because of the continued cycle of injury and the death of type II epithelial cells (Adamson *et al.*, 1988; Kasper and Haroske, 1996). The failure of re-epithelialisation leads to the loss of basement

membrane integrity, which results in the formation of a provisional matrix on the denuded basement membrane of the alveolar wall.

The epithelial injury is followed by subsequent recruitment of neutrophils and inflammatory cells, including monocyte, macrophage, lymphocytes and eosinophils into the lung (Keane *et al.*, 2005). During this stage, a number of pro-inflammatory and pro-fibrotic mediators (Table 1.3) are produced. These mediators then trigger the migration of fibroblasts to the site of injury, and stimulate fibroblasts to proliferate, differentiate to myofibroblasts with the deposition of collagen and other extracellular matrix (ECM) components into the provisional matrix. In normal wound healing, following deposition of limited ECM into the wound space, myofibroblasts contract to close the wound. Myofibroblasts and other effector cells including inflammatory cells then undergo apoptosis and are cleared from the wound space. Finally, type II epithelial cells proliferate to regenerate the epithelium. In pulmonary fibrosis, however, the abnormal apoptosis in these important effector cells exaggerates the process of pulmonary fibrosis (Thannickal and Horowitz, 2006a; Fattman, 2008). For example, studies have shown that fibroblast/myofibroblasts from patients with pulmonary fibrosis are not only resistant to apoptosis; they also induce apoptosis of human AEC by secreting soluble factors such as angiotensin peptide (Uhal *et al.*, 1995; Wang *et al.*, 1999). Additionally, inflammatory cells such as macrophages appear to have a prolonged survival time (Flaherty *et al.*, 2006). Conversely, epithelial cell apoptosis is increased which leads to the further damage of basement membrane integrity and facilitates the migration of fibroblasts (Kuwano, 2007). Without restoration of the alveolar epithelium, the invasion of fibroblasts and myofibroblasts continues which results in excessive matrix deposition and finally the interstitial fibrosis.

In summary, it is generally held that acute or repeated lung injury leads to the loss of type I epithelial cells, proliferation of type II epithelial cells; recruitment of inflammatory cells with the release of pro-inflammatory and pro-fibrotic mediators. This causes the migration, proliferation and differentiation of fibroblasts, which leads to the formation of fibroblastic foci and results in the dramatic deposition of ECM in the lung parenchyma. It is thought that these events occur in a sequential, yet dynamic overlapping manner (reviewed in (Keane *et al.*, 2005; Keane *et al.*, 2006).

1.2.1 Role of inflammation in pulmonary fibrosis

As mentioned in **Section 1.2** the theory of the pathogenesis of pulmonary fibrosis has shifted during the last decade, and a major debate surrounded is still about the role of

inflammation in pulmonary fibrosis, particularly in patients with IPF. Evidence for the critical involvement of inflammation in pulmonary fibrosis was mainly based on observations obtained with animal models of pulmonary fibrosis, where inflammation plays a critical role in the development of pulmonary fibrosis. However, this notion has now been challenged and the contribution of inflammation to the development of all forms of pulmonary fibrosis remains a controversial issue.

It has been suggested by Selman and colleagues that inflammation plays little or no role in the pathogenesis of IPF (Selman *et al.*, 2001). Selman and colleagues also proposed that pulmonary fibrosis is rather a result of epithelial micro-injury and abnormal wound healing with cross-talk between epithelial and fibroblastic cells. This is based on the observations that only mild inflammation is seen in lung sections from patients with IPF. This theory is also supported by studies in some animal models of lung fibrosis. For example, over-expression of the potent pro-fibrotic mediator TGF-β has been found to cause progressive fibrosis in mice without any significant involvement of inflammation (Sime *et al.*, 1997). In addition, mice lacking the αvβ6 integrin, a critical component for TGF-β activation, develop significant inflammation, but fail to progress to fibrosis after bleomycin injury. It therefore seems that inflammation may not be necessary to the development of fibrosis. As mentioned previously in Section 1.1, this may explain why anti-inflammatory treatment has no beneficial effect for patients with pulmonary fibrosis (Raghu, 2006). In conclusion, the evidence mentioned above suggests that inflammation is a minor participant for the development of some forms of lung fibrosis.

This hypothesis is criticized by researchers who believe that this theory has fundamentally ignored the natural history of the pathogenesis of UIP and the "so-called evidence" is not convincing (Strieter, 2002). First, this notion is based on observations from single biopsy specimen, and these biopsies represent established disease with evidence of end-stage fibrosis. Second, in studies with over-expression of TGF-β in mice, supply of superphysiological concentrations of TGF-β has bypassed inflammation-induced TGF-β expression. As suggested by studies by Lawrence and colleagues, TGF-β is necessary for the resolution of inflammation (Lawrence *et al.*, 2001), it is therefore possible that expression of physiologically relevant TGF-β may not lead to fibrosis. Indeed, studies with transient overexpression of IL-1β have demonstrated that the sustained production of TGF-β and the development of fibrosis are caused by local production of proinflammatory cytokines and a vigorous inflammatory response (Kolb *et al.*, 2001). This suggests that inflammation is important

in initiating the fibrotic response but, perhaps, not necessary to sustain this process. Finally, the failure of anti-inflammatory treatment may be due to the fact that the patients have already developed end-stage disease. In contrast, Flaherty and colleagues (Flaherty *et al.*, 2001b) strongly propose that inflammation is a necessary event in pulmonary fibrosis based on observations that the patterns of UIP and NSIP between lobes co-exist in the same patient in 26% of all patients investigated. This study supports the concept of an evolving process from inflammation to fibrosis since NSIP is characterized by chronic interstitial inflammation. Animal models of fibrosis also demonstrate that the inflammatory response precedes the fibrotic response (Chandler, 1990; Christensen *et al.*, 1999). In addition, an influx of inflammatory cells has been detected in bronchoalveolar lavage fluid (BALF) and in lung biopsies from patients with IPF during the exacerbation period, indicating the role of inflammation in pulmonary fibrosis (Parambil *et al.*, 2005; Kim *et al.*, 2006). A very recent review by Bringardner and colleagues proposed that inflammation plays a critical, but untraditional role in pulmonary fibrosis (reviewed in (Bringardner *et al.*, 2008). In conclusion, it is likely that inflammation and fibrotic responses may both be important mechanisms leading to self-perpetuating fibrosis.

1.3 Mediators of pulmonary fibrosis

A number of mediators such as cytokines, growth factors and coagulation proteinases have been shown to either directly or indirectly be involved in the fibrotic process. These cytokines and growth factors, produced by multiple cell types, including epithelial, endothelial cells, fibroblasts/myofibroblasts and inflammatory cells during the process of pulmonary fibrosis, can in turn activate and mediate interactions between these cells as shown in Table 1.3. The following section briefly describes the mediators currently known to have the greatest impact on pulmonary fibrosis.

1.3.1 Transforming growth factor-β (TGF-β)

There are three distinct TGF-β isoforms (TGF-β_{1-3}) in humans and TGF-β_1 is the most widely examined isoform in the context of experimental lung fibrosis (Eickelberg *et al.*, 1999). TGF-β_1 is one of the key profibrotic mediators identified in animal models and patients with fibrotic lung disease (Khalil *et al.*, 1991; Khalil *et al.*, 1996). Increased TGF-β mRNA and protein levels have been demonstrated in IPF (Broekelmann *et al.*, 1991). It is important to note that TGF-β localization may vary according to the disease stage, with localization to macrophages in early stage and to type II pneumocytes and fibroblastic foci at later stages (Khalil and Greenberg, 1991). TGF-β is the most potent inducer of collagen production characterised to date, and it promotes the differentiation

of fibroblasts into highly-activated myofibroblasts (Zhang *et al.*, 1996), it also plays a role in the differentiation of myofibroblasts from alveolar epithelial cells (Willis *et al.*, 2005). Moreover, in animal models of pulmonary fibrosis, TGF-β mRNA levels correlate with collagen expression (Hoyt and Lazo, 1988) and overexpression of TGF-β by adenoviral gene transfer leads to prolonged and severe interstitial and pleural fibrosis (Sime *et al.*, 1997). Finally, TGF-β may also contribute to fibrosis by inducing AEC apoptosis (Flaherty *et al.*, 2004).

1.3.2 Connective tissue growth factor (CTGF)

CTGF is highly expressed in the bleomycin model of lung fibrosis (Lasky *et al.*, 1998) and in patients with IPF (Kelly *et al.*, 2006). Although the mechanisms by which CTGF contributes to the development of lung fibrosis is currently poorly understood, there is evidence that CTGF exerts pro-fibrotic effects by directly stimulating fibroblast function and overexpression of CTGF has been shown to induce a mild and transient fibrotic response in mice (Bonniaud *et al.*, 2003). In addition, CTGF may also contribute to fibrosis by acting as a downstream mediator of TGF-β, with a particular role in stimulating fibroblast matrix production and differentiation (reviewed in (Leask and Abraham, 2003)).

1.3.3 Tumour Necrosis Factor-α (TNF-α)

TNF-α is a potent pro-inflammatory and pro-fibrotic cytokine. *In vitro* studies have shown that TNF-α promotes inflammatory cell recruitment and the induction of other pro-inflammatory cytokines (Smith *et al.*, 1998). In human fibrotic lung disease, TNF-α expression is unregulated in the lungs of patients with IPF (Zhang *et al.*, 1993). In animal models, overexpression of TNF-α results in an initial inflammation followed by fibrogenesis with TGF-β upregulation, fibroblast accumulation and extracellular matrix deposition (Sime *et al.*, 1998). However, TNF-α may play a dual role in fibrosis as it appears to limit the fibrogenic response at later stages (Fujita *et al.*, 2003). In addition, TNF-α promotes normal and fibrotic fibroblast apoptosis (Frankel *et al.*, 2006).

Mediators		Major Biological Activities			
	Major Targets	**Proliferation**	**Chemotaxis**	**Collagen Synthesis**	**Fibrogenic**
TGFβ$_1$	Fibroblast	±	+	++	++
PDGF	Fibroblast	++	+	+	+
FGF2	Fibroblast	+	+	+	+
TGFα	Fibroblast	+	0	+	+
TNFα	Fibroblast	+	0	+	+
IL-1	Fibroblast	+	0	+	+
IL-4	Fibroblast	+	+	+	+
IL-5	Eosinophil	+	+	+	+
IL-10	Fibroblast	-	+	-	±
IL-13	Fibroblast	+	-	+	+
IGF-1	Fibroblast	+	-	+	+
IFNγ	Fibroblast	-	+	-	-
IL-8	Macrophage	+	-	+	+
CCL2/MCP-1	Macrophage	-	+	+	+
MIP-1α	Macrophage	-	+	+	+
CTGF	Fibroblast	+	+	+	+
Thrombin	Fibroblast	++	+	+	++
FXa	Fibroblast	++	n/k	+	++

Table 1.3 Major mediators and their potential roles in lung repair and fibrosis (Modified and updated from (Gharaee-Kermani *et al.*, 2007)). ++, major role or strongly stimulatory; +, stimulatory; -, inhibitory; ±, variable-concentration-dependent; 0, no effect; n/k, not known.

1.3.4 Th1 and Th2 cytokines

There is accumulating evidence indicating that a shift from a type 1 (Th1: IL-2, IL-12, IL-18, IFN-γ) to a type 2 (Th2: IL-4, IL-5, IL-10, IL-13) cytokine profile is likely to be a key event in the progression of inflammation to fibrosis (Thannickal *et al.*, 2004). Th2 cytokines are pro-fibrotic by activating fibroblasts (Piguet *et al.*, 1996; Gharaee-Kermani and Phan, 2001). Th2 cytokines are linked to lung fibrosis in animal models,

although a functional link in humans is still lacking (Antoniou *et al.*, 2005). The opposing effects of Th1 and Th2 cytokines in fibrosis are supported by several investigations. For instance, Th2 cytokines such as IL-4 and IL-13 promote fibrosis by stimulating fibroblast collagen synthesis (Oriente *et al.*, 2000; Saito *et al.*, 2003), whereas the Th1 cytokine IFN-γ inhibits fibroblast function and procollagen synthesis (Diaz and Jimenez, 1997).

As the work in this thesis is focused on thrombin-induced CCL2 release, the role of thrombin and CCL2 in the pathogenesis of pulmonary fibrosis will be described in detail in **Section 1.5 and Section 1.8**, respectively.

1.4 Fibroblasts and pulmonary fibrosis

As already alluded to, fibroblasts migrate to the site of injury and produce extracellular matrix under the control of a host of mediators (Table 1.3) produced by inflammatory cells, injured and regenerating epithelial cells and fibroblasts themselves. Fibroblasts/myofibroblasts are major source of ECM, and the continued invasion of fibroblasts with enhanced ability of ECM generation play a critical role in accelerating the process of pulmonary fibrosis.

1.4.1 Origin of fibroblasts in pulmonary fibrosis

Fibroblasts are abundant cells in the lung. It has been reported that fibroblasts account for 35-50% of cells in the lung interstitium, and this number increases in the lungs of patients with pulmonary fibrosis (Jordana *et al.*, 1994). Embryologically, fibroblasts are believed to be of mesenchymal origin and were originally thought to replenish themselves by proliferation of local fibroblast populations resident in the lung parenchyma during wound healing (Serini and Gabbiani, 1999). In recent years, however, this notion has been challenged. More recent studies suggest that fibroblasts may be derived from a variety of other sources, at least following injury and in fibrotic disease. These sources include epithelial cells that undergo transdifferentiation to form fibroblasts by a process known as epithelial-mesenchymal transition (EMT), as well as circulating fibrocytes and bone marrow-derived mesenchymal stem cells (Abe *et al.*, 2001; Kalluri and Neilson, 2003; Phillips *et al.*, 2004).

1.4.1.1 Epithelial-mesenchymal transition

More than 20 years ago, studies by Greenburg and Hay first proposed the possible interconversion between epithelial and mesenchymal cells based on the evidence that epithelial cells in culture acquire mesenchymal features (Greenburg and Hay, 1982).

This process is now well-recognized as EMT. EMT is a process whereby epithelial cells lose their characteristic markers such as E-cadherin and zona occludens-1 resulting in the subsequent loss of polarity. Consequently, the epithelial cells become non-polarized with remodeled mesenchymal stress fiber and acquire mesenchymal makers such as fibroblast specific protein (FSP)-1 and α-smooth muscle actin (α-SMA) (Zavadil and Bottinger, 2005). Emerging evidence has now pointed to a role for EMT in fibrotic lung diseases such as IPF (Kalluri and Neilson, 2003). It has been shown that AEC can undergo EMT in response to TGF-β_1 stimulation (Willis *et al.*, 2005), and this has also been shown in experimental animal studies where epithelial-derived mesenchymal cells in TGF-β_1-induced fibrosis have been identified (Kim *et al.*, 2006). More importantly, studies have identified cells in IPF biopsy samples that co-express epithelial and mesenchymal markers, providing strong evidence of EMT during the development of lung fibrosis (Willis and Borok, 2007).

1.4.1.2 Fibrocytes in pulmonary fibrosis

Fibrocytes were first described over a decade ago as a population of cells with fibroblast-like properties (Bucala *et al.*, 1994). Fibrocytes are defined as circulating stem cells derived from bone marrow, which express both leukocyte and mesenchymal markers, including CD45, CD34 and collagen I (Bucala *et al.*, 1994; Moore *et al.*, 2005). These cells are spindle-shaped and distinguished from monocytes/macrophages, dendritic cells and B cells by the lack of specific marker protein expression. Fibrocytes represent approximately 0.5% of the peripheral blood leukocyte population and can produce fibroblast specific products, such as collagen I, III, and fibronectin (Abe *et al.*, 2001).

These circulating fibrocytes may serve as a source of fibroblasts in the lung and the recruitment of fibrocytes to the lung may be an important factor in the development of fibrotic responses, although this is still controversial (Gomperts and Strieter, 2007). Fibrocytes have been shown to transit into fibroblasts in culture, suggesting that these cells may serve as the circulating precursors for fibroblasts (Schmidt *et al.*, 2003; Phillips *et al.*, 2004; Moore *et al.*, 2005). A pathogenic role for fibrocytes in pulmonary fibrosis has been postulated from experimental models in which blockade of fibrocyte recruitment is protective following both bleomycin and fluorescein isothiocyanate challenge (Phillips *et al.*, 2004; Moore *et al.*, 2005; Moore *et al.*, 2006). Evidence to support the role of fibrocytes in patients with fibrotic interstitial lung disease has been recently provided by Mehrad and colleagues (Mehrad *et al.*, 2007). This study demonstrated that the number of fibrocytes in peripheral blood has been upregulated in

patients compared with healthy volunteers, suggesting that fibrocytes may be involved in the process of pulmonary fibrosis in humans.

It is important to note that fibrocytes express a number of chemokine receptors, including CCR2, CCR7, and CXCR4. The recruitment of fibrocytes to the site of injury is therefore likely mediated via chemokine and chemokine receptor interaction. Indeed, studies have demonstrated that inhibition of CCR2, CCR7, and CXCR4 has been shown to be protective in experimental lung fibrosis model and is accompanied by a reduction of the number of fibrocytes, suggesting that these receptors mediated signals may all serve to recruit fibrocytes to the lung (Abe *et al.*, 2001; Schmidt *et al.*, 2003; Phillips *et al.*, 2004; Moore *et al.*, 2005). Of particular interest, CCR2 is a receptor for CCL2, CCL7 and CCL12, in which CCL12 is only found in mouse and it is the functional homologue of human CCL2 (Moore *et al.*, 2006). As CCL2 is the main focus of this thesis, the role of CCL2 in fibrocyte recruitment will be discussed in more detail **in Section 1.8**.

Although fibroblasts can be derived from a variety of sources, the relative contribution of each of these sources to the fibrotic response are currently a topic of intense debate due to the potential implications for therapy in would healing and fibrosis.

1.4.2 Fibroblast function in pulmonary fibrosis

1.4.2.1 Extracellular matrix deposition

Fibroblasts are primarily responsible for maintaining the structural integrity of the airways and lung parenchyma by producing a variety of ECM components, such as collagen, laminin, fibronectin, hyaluronic acid and proteoglycans. In pulmonary fibrosis, these components are upregulated by mechanisms that alter the synthetic and degradative processes responsible for increased collagen deposition by fibroblasts. For example, increased number of fibroblasts at the site of injury leads to the excessive deposition of ECM. This may be caused by enhanced fibroblast migration as suggested by studies showing that fibroblasts derived from fibrotic lungs exhibit an enhanced migratory phenotype (Suganuma *et al.*, 1995). Increased fibroblast numbers at sites of injury may also be the consequences of increased proliferation and reduced apoptosis. Studies have demonstrated that fibroblasts derived from fibrotic lungs show higher proliferating capacity and are more resistant to apoptosis when compared with fibroblasts derived from normal lung (Raghu *et al.*, 1988; Ramos *et al.*, 2001; Moodley *et al.*, 2004). In addition, fibroblasts/myofibroblasts isolated from IPF lungs exhibit an increased synthetic capacity for collagen and other ECM components compared to

fibroblasts from normal lung (Hetzel *et al.*, 2005). As already mentioned in **Section 1.3,** a number of mediators are released after lung injury and these mediators stimulate fibroblasts to transdifferentiate into contractile, activated myofibroblasts, which synthesize and deposit abundant ECM proteins, such as type I and III collagens (Phan, 2002). These mechanisms all contribute to the excessive deposition of ECM in the fibrotic lung.

ECM degradation is regulated by matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs). MMPs are responsible for ECM degradation, whilst TIMPs are endogenous inhibitors of MMPs. In pulmonary fibrosis, the imbalance between MMPs and TIMPs is another important mechanism leading to the excessive deposition of ECM. Studies have shown that TIMPs are more widely distributed than MMPs in the lungs from IPF patients (Selman *et al.*, 2000). Furthermore, there is decreased production of MMPs and increased production of TIMPs by fibroblasts derived from IPF patients compared with fibroblasts derived from normal lung (Selman *et al.*, 2000; Ramos *et al.*, 2001). These studies suggest that there is a prevailing non-degradative microenvironment in the lungs of patients with IPF.

1.4.2.2 Production of inflammatory and fibrotic mediators

Fibroblasts were thought to be passive participant in tissue repair through their end stage production of extracellular matrix proteins. Although the elaboration of matrix is a key factor in fibrosis, the roles of fibroblasts and myofibroblasts likely extend beyond the production of ECM proteins. It appears that these mesenchymal cells are not just the primary cellular source of collagen, but also a major source of inflammatory and fibrogenic cytokines in pulmonary fibrosis (Jordana *et al.*, 1994). These cells express fibrogenic cytokines, chemokines, growth factors and their receptors, adhesion molecules, and are able to recruit and activate inflammatory cells (please see Table 1.3) (Phan, 2002). For example, IL-8, the member of CXC chemokine family generated by macrophages, is an activator and chemoattractant for neutrophils. IL-8 has been identified as a marker implicated in the development of airway inflammation and a major contributor to the neovascularisation in fibrotic diseases (Lynch *et al.*, 1992; Bonfield *et al.*, 1995; Nocker *et al.*, 1996; Keane *et al.*, 1997). Surprisingly, studies have demonstrated that the major source of this important inflammatory chemokine was the interstitial fibroblast rather than the macrophage (Keane *et al.*, 1997), further supporting the important role of fibroblasts in inflammation.

In addition, fibroblasts may also contribute to the production of mediators via its interaction with other inflammatory and immune cells. There is abundant evidence that the interaction between fibroblasts and these cells is bi-directional. For example, macrophage inflammatory protein 1α (MIP-1α) production by macrophage was found to be markedly increased when these macrophages were co-cultured with fibroblasts (Steinhauser *et al.*, 1998). T cells can modulate fibroblast proliferation and collagen synthesis by generating IL-4. On the other hand, studies have demonstrated that fibroblasts can also mediate the activation state of T cells (Hogaboam *et al.*, 1998b). For example, enhanced release of adhesion molecules and cytokine production by T cells is caused by the interaction between fibroblasts and T cells, such as VCAM-1 and IL-6 (Bombara *et al.*, 1993).

Finally, generation of these mediators by fibroblasts directly or indirectly can in turn stimulate fibroblasts via autocrine mechanism which further exaggerates profibrotic effects of fibroblasts such as proliferation, differentiation and matrix deposition.

1.4.2.3 Cross-talk between fibroblasts and epithelial cells

Epithelial and mesenchymal cell interaction is important in lung development, and the tight regulation of epithelial and mesenchymal cell functions is necessary for normal alveolarization (Hogan and Yingling, 1998). Current hypotheses propose that abnormal interaction between epithelial cells and fibroblasts may play an important role in the pathogenesis of pulmonary fibrosis by preventing resolution of wound repair (reviewed in (Horowitz and Thannickal, 2006)). For example, re-epithelialisation after injury is essential for would healing and excessive AEC apoptosis interrupts this process and therefore plays a critical role in the pathogenesis of pulmonary fibrosis. Studies of IPF tissue have shown that AEC apoptosis is increased in cells adjacent to fibroblastic foci, suggesting that fibroblasts/myofibroblasts may play a role in epithelial cell apoptosis (Uhal *et al.*, 1998; Barbas-Filho *et al.*, 2001). Indeed, fibroblasts/myofibroblasts derived from patients with IPF have been found to induce human AEC apoptosis by secreting soluble factors such as angiotensin peptides (Uhal *et al.*, 1995; Wang *et al.*, 1999). In addition, excessive proliferation of fibroblasts contributes to fibrosis and this can be the result of reduced inhibitory effects of epithelial cells on fibroblasts. It is well known that cyclooxygenase-2 (COX-2) derived prostaglandin E2 (PGE_2) plays an important role in pulmonary fibrosis by inhibiting fibroblast proliferation and procollagen gene expression (Wilborn *et al.*, 1995; Moore *et al.*, 2003; Hodges *et al.*, 2004). Studies have demonstrated the reduced expression of COX-2 in bronchial epithelial cells in patients with IPF (Petkova *et al.*, 2003).

Taken together, these findings support the concept that the fibroblast is a major effector cell in mediating lung fibrosis by synthesizing ECM components and inflammatory mediators, as well as displaying abnormal interactions with epithelial cells.

1.5 Role of the coagulation cascade in pulmonary fibrosis

The coagulation cascade is a complex and highly regulated proteolytic system with its prime function of generating insoluble, cross-linked fibrin strands which bind and stabilize the weak platelet haemostatic plug, formed at the sites of injury. The coagulation cascade is now gaining recognition as a multifunctional signalling system implicated in a number of physiological and pathophysiological events, including pulmonary fibrosis. There is increasing evidence that the coagulation cascade plays an important role in pulmonary fibrosis as activation of the coagulation cascade has been demonstrated to be a characteristic feature of several fibroproliferative lung disorders in humans and in animal models (Chapman *et al.*, 1986; Kotani *et al.*, 1995; Chambers, 2003, 2008).

1.5.1 Activation of the coagulation cascade

One of the earliest events following tissue injury is the activation of the coagulation cascade. The coagulation cascade is activated when damage to the vascular network of the lung occurs from both external and internal insults. The classical model of blood coagulation involves a cascade of zymogen activation reactions via two highly regulated pathways, namely the extrinsic and intrinsic coagulation pathways (Figure 1.2).

The coagulation proteinases of both pathways circulate in the plasma as inactive zymogens and the majority of zymogens are activated by limited proteolysis. At each stage, a precursor protein is converted to an active proteinase by cleavage of one or more peptide bonds in the precursor molecule. Both extrinsic and intrinsic pathways activate the "common pathway" via FX activation. The final proteinase generated is thrombin. Activated thrombin then mediates the aggregation of platelets and the conversion of fibrinogen to fibrin; hence a stable clot consisting of aggregated platelets enmeshed in fibrin is formed and temporarily plugs damaged vessels.

The extrinsic pathway is felt to be the main mechanism leading to the activation of the coagulation cascade *in vivo*. However, the final production of thrombin triggered by the extrinsic pathway is limited. In order to maintain coagulation, thrombin synthesized through the initial TF/FVIIa/FXa ternary complex catalyses the activation of FXI, FIX,

FVIII and FX. This leads to the initiation of intrinsic pathway which results in the prolonged generation of the prothrombinase complex, a major burst in thrombin activity, and subsequent sustained blood coagulation via dramatic amplification of the original signal (reviewed in (Chambers, 2008)). It is therefore concluded that initial activation of the extrinsic pathway following vascular injury generates limited amounts of thrombin, which in turn triggers the initiation of the intrinsic pathway where the coagulation is maintained by the dramatic amplification of the initial signal.

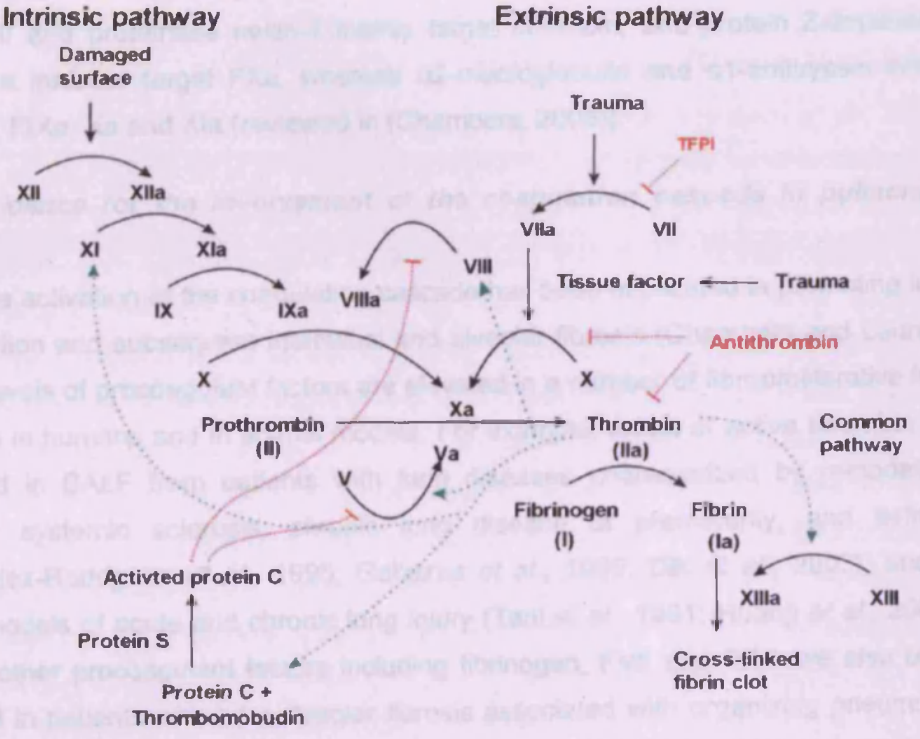


Figure 1.2 The coagulation cascade. This figure shows the activation of coagulation proteinases leading to the generation of thrombin via both the extrinsic and intrinsic pathways. TFPI: tissue factor pathway inhibitor. —|, the inhibitory pathway; ---▶, the positive feedback.

The coagulation cascade is tightly regulated by both negative feedback mechanisms as shown in figure 1.2, and by locally produced and circulating endogenous anticoagulants. The major anticoagulants include tissue factor pathway inhibitor (TFPI), antithrombin, and the thrombin-thrombomodulin protein C system. The major proteinase inhibitor of the extrinsic pathway is TFPI which targets the TF-VIIa-FXa

transient ternary complex. Antithrombin regulates the intrinsic pathway by irreversibly neutralizing serine proteinases in the presence of heparin. The thrombin-thrombomodulin-protein C system is initiated when thrombin binds to thrombomodulin on the vascular endothelium. Thrombin then rapidly activates protein C to activated protein C (APC), which can be further enhanced by the activation of endothelial cell protein C receptor (EPCR). Following its subsequent release from the cell surface, APC binds to protein S and inactivates FVa and VIIIa in order to inhibit further thrombin generation. Other physiological inhibitors include heparin cofactor II, proteinase nexin-1, α2-macroglobulin, α1-antitrypsin, and protein Z-dependent proteinase inhibitor. Heparin cofactor II and proteinase nexin-1 mainly target thrombin, and protein Z-dependent proteinase inhibitor target FXa, whereas α2-macroglobulin and α1-antitrypsin inhibit thrombin, FIXa, Xa and XIa (reviewed in (Chambers, 2008)).

1.5.2 Evidence for the involvement of the coagulation cascade in pulmonary fibrosis

Excessive activation of the coagulation cascade has been implicated in promoting lung inflammation and subsequent interstitial and alveolar fibrosis (Chambers and Laurent, 2002). Levels of procoagulant factors are elevated in a number of fibroproliferative lung disorders in humans and in animal models. For example, levels of active thrombin are increased in BALF from patients with lung diseases characterized by remodeling, including systemic sclerosis, chronic lung disease of prematurity, and asthma (Hernandez-Rodriguez *et al.*, 1995; Gabazza *et al.*, 1999; Dik *et al.*, 2003), and in animal models of acute and chronic lung injury (Tani *et al.*, 1991; Huang *et al.*, 2001). Several other procoagulant factors including fibrinogen, FVII and FX have also been identified in patients with intra-alveolar fibrosis associated with organizing pneumonia (BOOP) (Peyrol *et al.*, 1990). Similarly, tissue factor VII/VIIa complexes have been identified in BALF from patients with ARDS (Idell *et al.*, 1989). In addition, TF expression is upregulated in the lungs of patients with IPF and systemic sclerosis (Imokawa *et al.*, 1997). More importantly, FX has recently been shown to be upregulated and locally produced in IPF patients in recent studies performed in our laboratory (Krupiczojc *et al.*, in revision).

A number of studies have raised the possibility that excessive activation of the coagulation cascade in fibrotic and proliferative lung diseases may be the consequence of decreased production of anti-coagulant factors. For example, decreased protein C activation is found to be associated with abnormal collagen turnover in patients with interstitial lung disease (Yasui *et al.*, 2000). There is also evidence that the protein C

pathway is deficient in the lungs of patients with IPF and sarcoidosis, as well as collagen vascular disease-associated interstitial lung disease (Kobayashi *et al.*, 1998). Plasma and intra-alveolar protein C levels are also decreased in patients with ALI/ARDS (Ware *et al.*, 2007). Similarly, levels of endogenous anticoagulants such as antithrombin in patients with ARDS have been found to be reduced (Gando *et al.*, 2003).

A central role for the coagulation cascade in the pathogenesis of pulmonary fibrosis has been demonstrated by studies showing that blockade of this cascade is protective in experimental lung fibrosis. For example, previous work in our laboratory has shown that direct thrombin inhibition leads to a significant reduction in lung collagen accumulation in the bleomycin model of lung injury and fibrosis (Howell *et al.*, 2001). Heparin, which inhibits local activation of coagulation proteinases by potentiating the formation of antithrombin III-serine proteinase complexes, has been shown to improve gas exchange in an animal model of acute lung injury and attenuate pulmonary fibrosis in the bleomycin mouse model (Piguet *et al.*, 1996; Abubakar *et al.*, 1998). Similarly, intratracheal instillation of the endogenous anticoagulant APC has also been shown to be protective in the bleomycin model (Yasui *et al.*, 2001). More recently, it was reported that intratracheal gene transfer of TFPI decreases bleomycin-induced thrombin generation and pulmonary fibrosis in rats (Kijiyama *et al.*, 2006). In humans, a recent multicenter study demonstrated that anti-coagulant treatment has a beneficial effect on survival in IPF patients (Kubo *et al.*, 2005). The patient selection in this study is controversial but the outcome supports the importance of the role of coagulation cascade in pulmonary fibrosis. Taken together, these data support the notion that TF-mediated coagulation in the extravascular intra-alveolar space is important and that anticoagulant therapy could be beneficial.

As previously mentioned, the coagulation cascade primarily promotes haemostasis at sites of vascular injury by generating a stable, insoluble, cross-linked fibrin blood clots. In pulmonary fibrosis, however, it mediates a number of cellular responses that may play critical roles in the pathogenesis of this disease (reviewed in (Chambers, 2008)). Most of these cellular effects are mediated by a unique family of ubiquitously expressed receptors, termed proteinase-activated receptors (PARs) (Vu *et al.*, 1991). These effects and receptors will be discussed in the forthcoming sections in detail.

1.6 Proteinase-activated receptors

The PARs belong to the superfamily of G-protein coupled receptors (GPCRs) with seven transmembrane domains, and are activated via proteolytic cleavage by proteinases (Dery *et al.*, 1998; Coughlin, 2000; Macfarlane *et al.*, 2001; Hollenberg and Compton, 2002). There are currently four members (PAR_{1-4}) in this family (Table 1.4). Collectively, the proteinases of the coagulation cascade can activate all four members of the PAR family. In addition, a recent report has suggested that PAR_1 can be activated by the matrix metalloproteinase, MMP1 (Boire *et al.*, 2005). The mechanisms for PAR activation will be discussed in detail in **Section 1.6.1**. Our current understanding on the major activators, pharmacology, expression patterns, and major G protein signalling pathways of the PARs is summarized in Table 1.4.

	PAR_1	PAR_2	PAR_3	PAR_4
Activating Proteinases	Thrombin, APC/EPCR, FVIIa/TF/FXa;Trypsin ;Plasmin; Cathepsin G;MMP-1	Trypsin, Tryptase, FVIIa/TF/FXa Matriptase/MT-serine	Thrombin Trypsin	Thrombin Trypsin, FVIIa/X Cathepsin G
Tethered ligand	SFLLRN (h) SFFLR (m, r)	SLIGKV (h) SLIGRL (m, r)	TFGRAP((h) SFNGGP(m)	GYPGQV(h) GYPGKV(m)
Antagonists	RWJ-5611 RWJ-58259 SCH 530348 SCH 79797	ENMD-1068	---	YD-3
Inhibitory proteinases	Cathepsin G Elastase, Chymase	Elastase, Chymase	Cathepsin G	Unknown
Chromosoal Location	5q13	5q13	5q13	19p12
Cleavage site	Arg41-Ser42	Arg36-Ser37	Lys38-Thr39	Arg47-Gly48
Hirudin-like binding site	Yes	No	Yes	No
PAR-Coupled-G proteins	$G_{q/11}$, $G_{i/o}$, $G_{12/13}$, $G_{\beta\gamma}$	$G_{q/11}$, G_i, $G_{12/13}$	G_q, G_o	$G_{12/13}$, G_q
Expression in the lung	Airways, blood vessels, lung parenchyma	Airways, blood vessels, bronchial glands, lung parenchyma	Airways	Airway, blood vessels, cardiovascular system

Table 1.4 Pharmacology and characteristics of proteinase-activated receptors (Macfarlane *et al.*, 2001; Steinhoff *et al.*, 2005; Chambers, 2008; Jeong *et al.*, 2008). The letters denote the amino acid sequences of peptides in one letter code: APC/EPCR, activated protein C and endothelial cell protein C receptor (cofactor) complex; h, human; m, mouse; r, rat.

1.6.1 Mechanisms of PAR activation

Most GPCRs are activated reversibly by small hydrophilic molecules to elicit cellular responses (Wettschureck and Offermanns, 2005). Unlike classical GPCRs, the PARs on the other hand are, in essence, peptide receptors that effectively carry their own ligands which are recognized by specific proteinases. These unique activation sites lie hidden until revealed by receptor cleavage. Cleavage of their extracellular N-terminus by proteinases generates a new N-terminus that acts as an intramolecular ligand by interacting with the second extracellular loop of the receptor. This induces a

conformational shape change, which enables the receptor to interact with heterotrimeric G proteins and initiate downstream signalling responses (Figure 1.3).

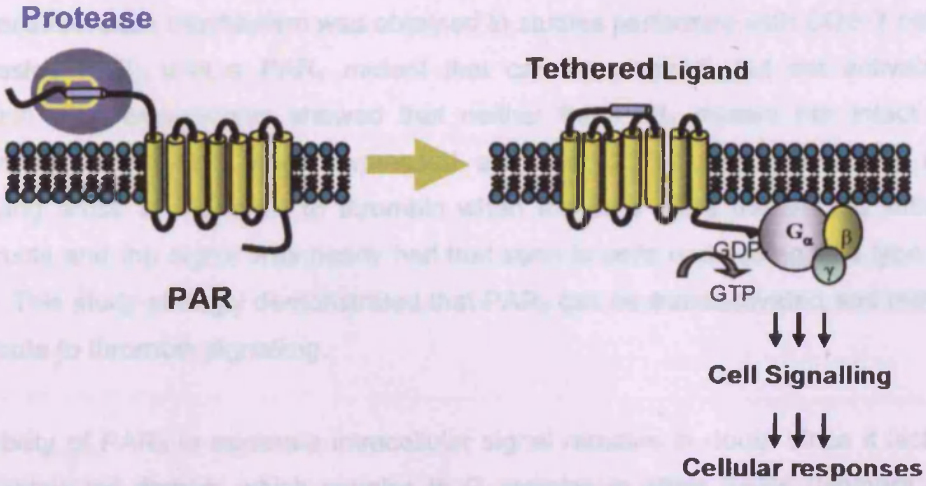


Figure 1.3 Mechanism of activation of PAR_1. Figure shows cleavage of a 41 amino-acid sequence from the N-terminus of the receptor by the coagulation proteinase binding to this region. An intrinsic tethered ligand is then unmasked and is able to interact with the second extracellular loop of the receptor. This in turn leads to a conformational change at the C-terminus followed by the association with heterotrimeric G-proteins. The interaction between the receptor and G-proteins initiates cell signalling and subsequently leads to a number of cellular responses.

The tethered ligand mechanism of activation is not the only feature to separate PARs from other GPCRs, the sequence of this proteolytically revealed tethered ligand has also become the most clear-cut molecular characteristic that distinguishes one PAR from another. This has lead to the development of short synthetic activating peptides corresponding to the tethered ligand integral to the receptor. These short synthetic peptides can experimentally activate PARs *in vitro* without the need for proteolysis (Vu *et al.*, 1991; Scarborough *et al.*, 1992). Although they are generally less efficient than the *in vivo* physiological activators (proteinases) of the PARs, these PAR-activating peptides have become valuable tools to identify the involvement of receptors in functional studies in systems where more than one PAR receptor is expressed.

Although activation of PAR receptors mainly involves the stimulation by proteinases, PAR receptors can also activate each other under certain circumstances. This intermolecular activation between PARs occurs when more than one PAR is present.

For example, PAR_2 is cleaved and activated by trypsin and tryptase. It cannot be activated by thrombin directly and was assumed to play no role in thrombin-triggered signalling events. This concept has now been challenged by studies suggesting that PAR_1 cleaved by thrombin can transactivate PAR_2 (O'Brien *et al.*, 2000). Evidence for this transactivation mechanism was obtained in studies performed with COS-7 cells co-expressing PAR_2 with a PAR_1 mutant that can be cleaved, but not activated by thrombin. The experiments showed that neither the PAR_1 mutant nor intact PAR_2 responded to thrombin when expressed alone in COS-7 cells. However, robust signalling arose in response to thrombin when the cells were transfected with both constructs and the signal was nearly half that seen in cells expressing wild type PAR_1 alone. This study strongly demonstrated that PAR_2 can be transactivated and may also contribute to thrombin signalling.

The ability of PAR_3 to generate intracellular signal remains in doubt since it lacks the cytoplasmic tail domain which couples to G proteins in other PARs (Ishihara *et al.*, 1997). However, increasing evidence suggests that PAR_3 can play a cofactor-like role in the cleavage of other PARs and participate in signalling. For example, in mouse platelets, PAR_3 was shown to assist efficient cleavage of PAR_4 by thrombin (Nakanishi-Matsui *et al.*, 2000). This study showed that co-expression of $mPAR_3$ (murine PAR_3) and $mPAR_4$ accelerated the rate of thrombin-induced $mPAR_4$ cleavage and $mPAR_4$ can be activated by thrombin at much lower EC_{50} than if $mPAR_4$ is expressed alone. In addition, PAR_3 can also function as an important allosteric modulator for PAR_1 signalling as suggested by recent studies by McLaughlin and colleagues (McLaughlin *et al.*, 2007). Using bioluminescent resonance energy transfer-2 ($BRET^2$), it was found that PAR_1 and PAR_3 may form constitutive heterodimers under physiological conditions. Upon activation, the $PAR_{1/3}$ heterodimers induce signalling distinct from $PAR_{1/1}$ homodimers by increasing the PAR_1-$G\alpha_{13}$ interaction. It is also worth mentioning that a most recent report demonstrated that PAR_3 can mediate thrombin-induced IL-8 release in human embryonic kidney (HEK) cells (Ostrowska and Reiser, 2008a). However, whether PAR_3 can signal on its own still remains controversial and further studies in other cell types, including fibroblasts are required.

The preferential activation of PARs may vary when a proteinase can activate multiple PARs and when these PARs are expressed at the same time. This variation has been seen for proteinases at different concentrations, such as thrombin, or in different cell types. For instance, both PAR_1 and PAR_4 can be activated by thrombin. However, PAR_4 is much less sensitive to thrombin, with an EC_{50} for the proteinase approximately

100-fold higher than the corresponding EC_{50} for PAR_1 (Major *et al.*, 2003). Activation of PAR_1 by thrombin is facilitated by the hirudin-like sequence towards the N-terminal end of PAR_1 which can interact with thrombin's anion-binding exosite. However, PAR_4 lacks a hirudin-like binding sequence. Moreover, both the N- and C- termini of PAR_4 are markedly different from the other receptors, as is the cleavage site. This may explain why thrombin is less efficient at activating PAR_4. It has been suggested that PAR_4 may serve as a co-receptor for PAR_1 by sustaining the cellular effects of thrombin when PAR_1 becomes rapidly inactivated (Shapiro *et al.*, 2000). In addition, recent studies in human platelets have reported the existence of PAR_1-PAR_4 complex (Leger *et al.*, 2006). This study also provided evidence that PAR_4 cleavage is accelerated on coexpression with PAR_1, and its activity is markedly enhanced by thrombin-PAR_1 interaction.

1.6.2 Regulation of PAR signalling

Activation of PARs by proteinases is an irreversible process and the tethered ligand will not diffuse away, which may therefore enable continuous activation. Once activated, the PARs need to be turned off to prevent indefinite signalling. On the other hand, the PARs also have to be replaced to re-establish sensitivity to proteinases. A number of mechanisms may contribute to the termination of PAR signalling. These include receptor inactivation, receptor desensitization, and receptor endocytosis (Dery *et al.*, 1998; Hollenberg and Compton, 2002; Ossovskaya and Bunnett, 2004).

1.6.2.1 Receptor inactivation

As shown in Table 1.4, PARs may be subject to proteolytic inactivation by proteinases such as cathepsin G and neutrophil elastase. These proteinases inactivate PAR_1 by cleaving the receptor at a specific site between the C-terminus and its tethered ligand, thus amputating the ligand from the receptor body. These deactivated receptors are refractory to proteinase activation, but still react to activating peptides (Renesto *et al.*, 1997).

1.6.2.2 Receptor desensitization

Receptor desensitization involves uncoupling the receptor from its G proteins and the downstream signalling pathways (Bohm *et al.*, 1996). In this process, receptor-G protein interaction is effectively interrupted by receptor phosphorylation which leads to the termination of signal transduction. This process is mediated via two mechanisms involving in two classes of protein kinases. One is homologous receptor desensitisation which involves agonist-dependent phosphorylation of specific C-terminal residues of

the receptor by G protein receptor kinases (GRKs) such as GRK3 (Ishii *et al.*, 1994). These GRKs utilise proteins known as β-arrestins as cofactors. The other mechanism is heterologous receptor desensitisation that occurs as a result of C-terminal phosphorylation by other intracellular protein kinases such as protein kinase C and A (Yan *et al.*, 1998).

1.6.2.3 Receptor endocytosis and cycling

Following activation, PARs are recruited into clathrin-coated pits and internalised by endocytosis to be degraded intracellularly (Hoxie *et al.*, 1993). Most of the internalized receptors are transferred to lysozomes rather than being recycled to the plasma membrane, like other GPCRs (Trejo and Coughlin, 1999). It has been shown that the process of lysosomal sorting is regulated by the cytoplasmic tail of PAR_1 (Brass *et al.*, 1994). It is also worth mentioning that some cleaved receptors are found to traffic back to the cell surface, and remain unresponsive to the same proteinase. However, these receptors are still sensitive to activation by the PAR activating peptides such as SFLLR in *in vitro* studies (Hammes and Coughlin, 1999).

1.6.2.4 Receptor resensitisation

The ability of a cell to regain responsiveness to PAR activation depends on both the replacement of new receptors from the intracellular receptor stores and the synthesis of new receptors (Dery *et al.*, 1998). These mechanisms are cell-specific. For instance, cleaved PAR_1 cannot be replaced in platelets because they lack an intracellular pool of intact receptors, and lack the ability to synthesize large amounts of protein (Coughlin, 2000). Hence, platelets cannot regain responsiveness following thrombin stimulation (Coughlin, 1999). In contrast, abundant intracellular stores of PARs have been reported in endothelial cells (Horvat and Palade, 1995), fibroblasts (Hein *et al.*, 1994), and epithelial cells (Kong *et al.*, 1997). These receptors are protected from extracellular proteinases and can be located to the cell surface to replace cleaved receptors without the delay needed to synthesize new receptors, although in all cell types, the major mechanism for receptor replenishment after prolonged exposure to activating proteinases is new receptor synthesis.

1.7 Proteinase-activated receptor-1 (PAR_1)

PAR_1, the main high-affinity thrombin receptor, was the first PAR to be cloned and characterized. PAR_1 can also be activated by FXa, the ternary complex, TF/FVIIa/FXa (Riewald *et al.*, 2001), APC (Riewald *et al.*, 2002), and plasmin (Mandal *et al.*, 2005). The efficient activation of PAR_1 by thrombin is facilitated by a hirudin-like binding

domain C-terminus to the tethered ligand at N-terminus of PAR_1. The attraction between this domain and thrombin's anion binding exosite enables thrombin to effectively complex with PAR_1 and thus initiate cleavage of PAR_1. In contrast, efficient activation of PAR_1 by FXa is facilitated by FXa complexing with TF and FVIIa at the site of its generation, which enables FXa to localize to the cell surface. In this regard, FXa is five times more potent at activating PAR_1 compared to FXa alone (Riewald *et al.*, 2001). Similarly, APC utilizes cell surface EPCR as a cofactor to facilitate its activation of PAR_1 (Riewald *et al.*, 2002).

PAR_1 is widely distributed on a number of cell types, including human platelets, endothelial cells, epithelial cells, fibroblasts, smooth muscle cells, monocytes, lymphocytes, mast cells, T cells positive for CD8, CD16, and either CD56 or CD57, natural killer cells, CD34+ haematopoietic progenitor cells, dental pulp cells, neurons, glial cells, mast cells, and certain tumour cell lines (Steinhoff *et al.*, 2005). Previous studies have shown that PAR_1 knockout (KO) mice are protected from experimentally-induced glomerulonephritis (Cunningham *et al.*, 2001), arthritis (Yang *et al.*, 2005), and cerebral infarction (Junge *et al.*, 2003). PAR_1 antagonism was also found to be protective against experimental liver fibrosis (Fiorucci *et al.*, 2004). These studies clearly support the critical role of PAR_1 in mediating responses to tissue injury. In contrast, one study showed that PAR_1 KO mice are not protected in mouse models of endotoxemia where multiple PARs (PAR_{1-4}) appear to be important for the crosstalk between inflammation and coagulation (Pawlinski *et al.*, 2004). It is therefore likely that the contribution of PAR_1 to tissue inflammatory and fibrotic responses is both injury- and organ-specific. However, emerging evidence suggests that PAR_1 contributes to pulmonary fibrosis and PAR_1 may represent an attractive novel target for therapeutic intervention for both acute and chronic lung injury (reviewed in (Chambers, 2008)).

1.7.1 PAR_1 in pulmonary fibrosis

Supporting evidence for the importance of PAR_1 in pulmonary fibrosis has been provided by a recent finding from our laboratory that PAR_1 actively participates in acute inflammation and the chronic fibrotic phase of bleomycin-induced pulmonary fibrosis (Howell *et al.*, 2005). However, establishing the importance of this signalling receptor to human fibrotic lung disease remains challenging. Supportive evidence for the role of PAR_1 in human fibrotic lung disease was provided by the findings that PAR_1 expression is up-regulated in lung tissues of patients with IPF (Howell *et al.*, 2001). Studies by Bogatkevich and colleagues have also shown that PAR_1 is upregulated in lung tissues of patients in early and late stages of pulmonary fibrosis associated with scleroderma

(Bogatkevich *et al.*, 2005). In both cases, the upregulation of PAR_1 was attributed to resident alveolar macrophages and to fibroproliferative foci where fibroblasts accumulate. The critical role of PAR_1 in human fibrotic lung diseases is also supported indirectly by studies employing cultured primary lung fibroblasts. Studies have shown that in cultured airway cells under normal conditions, PAR_1 is the predominant receptor expressed by primary adult lung fibroblast (Sokolova *et al.*, 2005), and endothelial cells (Fujiwara *et al.*, 2004), indirectly suggesting the importance of PAR_1 in the pathological situations in the lung since these cells are important effector cells in the process of pulmonary fibrosis.

There is a wealth of evidence suggesting that PAR_1 is the major receptor involved in mediating thrombin's pluripotent cellular effects in the pathogenesis of pulmonary fibrosis (Chambers and Laurent, 2002; Howell *et al.*, 2002; Chambers, 2008). PAR_1 is a major focus of the work performed in this thesis and will be discussed in greater depth in forthcoming sections.

1.7.2 PAR_1-mediated cellular effects of thrombin in pulmonary fibrosis

PAR_1 plays an important role in normal tissue repair following injury by mediating a number of thrombin-mediated cellular effects, such as platelet aggregation, influencing endothelial and epithelial cell permeability, inflammatory cell recruitment, mesenchymal cell migration, proliferation and ECM deposition (Chambers, 2003, 2008).

1.7.2.1 Platelet aggregation

A large number of studies have confirmed that thrombin is a major agonist for platelets, initiating a series of events leading to platelet aggregation *in vitro* and *in vivo* (Eidt *et al.*, 1988). Platelet aggregation is a key process in hemostasis and thrombus formation and has been found to be correlated with lung inflammation and collagen deposition induced by bleomycin (Piguet and Vesin, 1994). The interaction of thrombin and platelets ensures the rapid formation of haemostatic plugs at sites of vascular injury. The interaction also enables thrombin to indirectly control the activity of the cells at the site of injury by triggering the release of a great variety of mediators from platelets immediately after injury. These mediators include thromboxane A2, platelet factor-4, PDGF (Hart *et al.*, 1990; Soma *et al.*, 1992) and TGF-β1 (Schini-Kerth *et al.*, 1997). Thrombin also mediates the translocation of P-selectin and CD40 ligand to the plasma membrane of platelets, facilitating binding to endothelial cells (Henn *et al.*, 1998).

Many of these effects can be mimicked with PAR_1 selective agonists and blocked with PAR_1 neutralising antibodies, suggesting a central role for PAR_1 in mediating these responses. However, studies have proved that some of the effects of thrombin on platelets can also be mediated by PAR_4 in human platelets. As mentioned previously, PAR_4 can mediate human platelet activation in response to higher concentrations of thrombin (Kahn *et al.*, 1999). Interestingly, thrombin mediates murine platelet aggregation via activation of PAR_3 and PAR_4 (Kahn *et al.*, 1998), rather than PAR_4 alone. In this system, murine platelets only express PAR_3 and PAR_4, PAR_3 does not mediate cellular effects directly, rather, it acts as a cofactor for the effective cleavage and activation of PAR_4 (Nakanishi-Matsui *et al.*, 2000).

1.7.2.2 Disruption of endothelial and alveolar membrane barrier function

Activation of PAR_1 is thought to contribute to the development of fibroproliferative lung disease by increasing pulmonary vascular permeability via an endothelial-dependent mechanism. This is thought to be a major mechanism leading to leak of coagulation proteinases and intra-alveolar fibrin deposition (Idell, 2003). PAR_1 activation on endothelial cells by thrombin promotes the expression of tissue factor (Bartha *et al.*, 1993), and adhesion molecules such as E- and P-selectin and intracellular adhesion molecule (ICAM)-1 (Sugama *et al.*, 1992; Shankar *et al.*, 1994). These molecules can further promote the initiation of coagulation and platelet adhesion and can also attract inflammatory cells to sites of injury. At higher concentrations, thrombin increases vascular permeability by influencing endothelial cell shape and disrupting endothelial cell-cell junctions via activation of PAR_1 (Lum *et al.*, 1993; Rabiet *et al.*, 1996). Consistent with this observation, direct systemic infusion of thrombin increases pulmonary microvascular permeability in the lungs of experimental animals (Lo *et al.*, 1985). Furthermore, microvascular permeability is abrogated in lungs isolated from PAR_1 KO mice after systemic perfusion with thrombin (Vogel *et al.*, 2000). Platelet adhesion is also thought to contribute to increased endothelial permeability by releasing serotonin (Henn *et al.*, 1998). Platelet adhesion can be triggered by PAR_1 activation at low thrombin concentrations. Conversely, activation of PAR_1 may also promote the recovery of endothelial barrier integrity (Komarova *et al.*, 2007). The barrier-protective effect of PAR_1 is mediated by the activation of sphingosine-1-phosphate 1 ($S1P_1$) in an autocrine or paracrine manner as a consequence of sphingosine kinase-1 (SphK-1)-mediated $S1P_1$ generation (Komarova *et al.*, 2007). Depletion of either SphK-1 or the $S1P_1$ receptor inhibits barrier enhancement induced by PAR_1 activation (Feistritzer and Riewald, 2005).

In addition to vascular permeability, PAR_1 activation by thrombin can also increase lung permeability by promoting epithelial cell apoptosis (Chin *et al.*, 2003). Activation of PAR_1 induced tight junctional zonula-occludens 1 disruption and apoptotic nuclear condensation in a caspase-3-dependent manner in epithelial cells, which ultimately led to the apoptosis of epithelial cells. As mentioned previously, excessive epithelial cell apoptosis leads to disruption of basement membrane integrity and facilitates the continuous migration of fibroblasts, which ultimately leads to the formation of fibroproliferative foci and the destruction of alveolar structure (reviewed in (Thannickal and Horowitz, 2006b; Fattman, 2008)).

1.7.2.3 Inflammatory cell recruitment

Although the contribution of inflammation to IPF remains controversial at the present time, there is compelling evidence that thrombin exerts potent pro-inflammatory effects and these effects are mainly mediated via PAR_1 (Sower *et al.*, 1995; Naldini *et al.*, 2002). Thrombin induces the release of potent pro-inflammatory mediators which have been implicated in the pathogenesis of pulmonary fibrosis, including CCL2, IL-6 and IL-8 (reviewed in (Chambers, 2008). Thrombin is a potent chemoattractant and mitogen for inflammatory cells (Grand *et al.*, 1996). For example, thrombin induces the expression of the T cell chemokine, RANTES, via PAR_1 in fibroblasts (Hirano *et al.*, 2002). Thrombin may further influence inflammatory cell trafficking by inducing the expression of endothelial cell adhesion molecules, including E-selectin, P-selectin and ICAM-1 via PAR_1-dependent mechanisms (Sugama *et al.*, 1992; Shankar *et al.*, 1994; Kaplanski *et al.*, 1997; Kaplanski *et al.*, 1998). These inflammatory mediators can in turn promote initiation and propagation of the coagulation cascade by upregulating the local expression of tissue factor (Leslie and Detty, 1986; van der Poll *et al.*, 1991; van der Poll *et al.*, 1994).

1.7.2.4 Pro-fibrotic effects

Thrombin is a potent pro-fibrotic mediator in pulmonary fibrosis. Thrombin is likely to play an important role in the fibroproliferative response to lung injury by exerting its potent mitogenic effects on lung fibroblasts (Trejo *et al.*, 1996) and its stimulatory effects on extracellular matrix production (Chambers *et al.*, 1998). Thrombin is mitogenic for fibroblasts *in vitro* and also *in vivo* (Dawes *et al.*, 1993; Ohba *et al.*, 1994). There is good evidence that these mitogenic and pro-fibrotic effects of thrombin are mediated via the activation of PAR_1. In support of this notion, previous studies conducted in host laboratory have shown that thrombin is present in BALF obtained from patients with pulmonary fibrosis associated with systemic sclerosis. This fluid is

furthermore mitogenic for cultured lung fibroblasts and this mitogenic activity can be blocked with the thrombin inhibitor, hirudin (Hernandez-Rodriguez *et al.*, 1995). Thrombin is capable of promoting the transformation of fibroblasts into α-SMA positive myofibroblasts, the predominant fibroblast phenotype present in active fibrotic lesions, by activating PAR_1 (Bogatkevich *et al.*, 2001). Finally, thrombin is also a chemoattractant for lung fibroblasts (Dawes *et al.*, 1993) and therefore may also contribute to fibrosis by recruiting fibroblasts to the site of lung injury. Additionally, the central role of PAR_1 in mediating FXa stimulatory effects on lung fibroblast function has also been established by studies performed in our laboratory (Blanc-Brude *et al.*, 2001). PAR_1 activation is also fibrotic by inducing apoptosis of epithelial cells (Suzuki *et al.*, 2005) as mentioned in **Section 1.4.2.3** (Pan *et al.*, 2001).

It should be noted that most of the PAR_1-mediated thrombin cellular effects occur via the generation of secondary mediators from a variety of cell types. Table 1.4 lists the major mediators and their documented cellular source. Further evidence that PAR_1 may be important in influencing both inflammation and fibrosis has been provided by studies performed in our and other laboratories showing that PAR_1 knockout mice are protected from bleomycin-induced lung injury (Howell *et al.*, 2005) and experimental liver fibrosis (Fiorucci *et al.*, 2004). PAR_1 is found to be highly expressed in the lungs of patients with IPF (Howell *et al.*, 2005) and in patients with pulmonary fibrosis associated with systemic sclerosis (Bogatkevich *et al.*, 2005). Taken together, these observations suggest that PAR_1 signalling in response to thrombin plays a central role in the pathogenesis of pulmonary fibrosis.

Mediators	Cellular source	Major effect
bFGF	Vascular smooth muscle cells (Stouffer and Runge, 1998)	Pro-fibrotic
CCL2	Monocytes (Colotta *et al.*, 1994) HUVEC (Marin *et al.*, 2001) Dermal fibroblasts (Bachli *et al.*, 2003) Hepatic stellate cells (Fiorucci *et al.*, 2004)	Pro-inflammatory and pro-fibrotic
CTGF	Lung fibroblasts (Chambers *et al.*, 2000) Epithelial cells (Riewald *et al.*, 2001)	Pro-fibrotic
Endothelin-1	Endothelial cells (Marsen *et al.*, 1995) Vascular smooth muscle cells (Lepailleur-Enouf *et al.*, 2000) Monocytes (Srivastava and Magazine, 1998)	Pro-fibrotic
ICAM-1/VCAM-1	HUVEC (Kaplanski *et al.*, 1998)	Pro-inflammatory
IGF-1	Vascular smooth muscle cells (Du *et al.*, 2001)	Pro-fibrotic
IL-1β	Macrophages/Monocytes (Naldini *et al.*, 2002)	Pro-inflammatory
IL-6	Lung fibroblasts (Sower *et al.*, 1995) Lung epithelial cells (Asokananthan *et al.*, 2002) Endothelial cells (Johnson *et al.*, 1998) Smooth muscle cells (Kranzhofer *et al.*, 1996) Monocytes (Naldini *et al.*, 2000)	Pro-inflammatory
IL-8	Lung fibroblasts (Ludwicka-Bradley *et al.*, 2000) Bronchial epithelial cells (Asokananthan *et al.*, 2002) HUVECs (Marin *et al.*, 2001) Monocytes (Asokananthan *et al.*, 2002)	Pro-inflammatory
PDGF	Alveolar macrophages (Tani *et al.*, 1997) Lung fibroblasts (Blanc-Brude *et al.*, 2005) Lung epithelial cells (Shimizu *et al.*, 2000) HUVEC (Shankar *et al.*, 1994) Vascular smooth muscle cells (Stouffer and Runge, 1998)	Pro-fibrotic
PGE_2	HUVEC (Houliston *et al.*, 2002) Colonic fibroblasts (Seymour *et al.*, 2003) Epithelial cells (Asokananthan *et al.*, 2002)	Pro-inflammatory and anti-fibrotic
RANTES	Synovial fibroblasts (Hirano *et al.*, 2002)	Pro-inflammatory
TGF-β1	Platelets (Soslau *et al.*, 1997) Vascular smooth muscle cells (Bachhuber *et al.*, 1997) Epithelial cells (Jenkins *et al.*, 2006)	Pro-fibrotic
TNF-α	Microglia (Suo *et al.*, 2002)	Pro-inflammatory and pro-fibrotic

Table 1.5 Pro-inflammatory and pro-fibrotic mediators induced in response to PAR_1 activation. HUVEC: human vascular endothelial cells.

1.7.3 PAR_1 signalling

1.7.3.1 G protein coupled-receptor signal transduction overview

Signal transduction systems enable cells to receive signals from extracellular stimuli and mount appropriate responses. Although a great number of receptors have been involved in detecting extracellular stimuli, the GPCRs have been so far regarded as the major receptor type for signal transduction and GPCRs represent the largest group of cell surface receptors encoded by the mammalian genome (>1% of human genes) (Wess, 1998). GPCRs such as PAR_1 have been implicated in regulating several cellular processes in the lung (reviewed in (Chambers, 2008)).

α-Subunits	Members of each α-subunits	Expression
$G\alpha_s$	$G\alpha_s$	Ubiquitous
	$G\alpha_{sXL}$	Neuroendocrine
	$G\alpha_{olf}$	Olfactory epithelium, brain
$G\alpha_{i/o}$	$G\alpha_{i1}$	Widely distributed
	$G\alpha_{i2}$	Ubiquitous
	$G\alpha_{i3}$	Widely distributed
	$G\alpha_o$	Neuronal, neuroendocrine
	$G\alpha_z$	Neuronal, platelets
	$G\alpha_{gust}$	Taste cells, brush cells
	$G\alpha_{t-r}$	Retinal rods, taste cells
	$G\alpha_{t-c}$	Retinal cones
$G\alpha_{q/11}$	$G\alpha_q$	Ubiquitous
	$G\alpha_{11}$	Almost ubiquitous
	$G\alpha_{14}$	Kidney, lung, spleen
	$G\alpha_{15/16}$	Hematopoietic cells
$G\alpha_{12/13}$	$G\alpha_{12}$	Ubiquitous
	$G\alpha_{13}$	Ubiquitous

Table 1.6 Heterotrimeric G proteins (Modified from (Wettschureck and Offermanns, 2005))

To understand the physiological actions of a given GPCR such as PAR_1 here, it is essential to identify the specific G proteins with which it is able to interact. G proteins are heterotrimeric guanine nucleotide-binding proteins which consist of α, β and γ subunits. Although there are many gene products encoding each subunit, four main classes of G proteins can be distinguished according to the primary sequences of their α subunits, as follows: $G\alpha_{i/o}$, $G\alpha_s$, $G\alpha_{q/11}$ and $G\alpha_{12/13}$ (each with different members,

Table 1.6). It should be pointed out that the $G\alpha_{15}$ and $G\alpha_{16}$ from the $G\alpha_{q/11}$ family are the murine and human versions of the same gene. In addition, there are 6 different G protein β and 12 distinct γ-subunits that have been described. The βγ complexes are extremely stable and usually regarded as one functional unit (Wess, 1998).

GPCRs such as PAR_1 interact with G proteins upon activation by upstream agonists. This interaction subsequently promotes the exchange of GDP for GTP on the Gα subunits, which leads to the dissociation of Gβγ from Gα. The free Gβγ and Gα subunits recognize and activate specific effector enzymes to initiate the desired physiological responses (Vauquelin and Van Liefde, 2005). G-protein deactivation is rate-limiting and occurs when the Gα subunit hydrolyzes GTP to GDP.

Investigation during the last few years demonstrated that the PAR_1-mediated effects mentioned above are transduced by various signaling pathways leading to diverse functions of PAR_1 under physiological and pathophysiological conditions. However, our understanding of signalling pathways involved in PAR_1-mediated cellular events is still far from complete. As a G protein-coupled receptor, PAR_1 exerts its multiple cellular effects by concomitant activation of several G proteins, including $G\alpha_{i/o}$, $G\alpha_q$ and $G\alpha_{12/13}$ as shown in figure 1.4 (Coughlin, 2000; McLaughlin *et al.*, 2005; Nguyen *et al.*, 2005). Detailed studies of PAR_1-mediated signal transduction in different cells and tissues have been reviewed by Steinhoff *et al* (Steinhoff *et al.*, 2005). This thesis will focus on the signalling pathways in the lung.

1.7.3.2 PAR_1-G protein signalling pathways in pulmonary fibrosis

PAR_1 is preferentially linked to $G\alpha_{q/11}$ and $G\alpha_{i/o}$ families, but it can also couple to $G\alpha_{12/13}$ with less efficiency (Wess, 1998). It is well-established that G-protein involvement in PAR_1 signalling is both cell- and tissue-specific. The forthcoming sections will be focused on PAR_1-G protein signalling in platelets, endothelial cells, epithelial cells and fibroblasts as these PAR_1-mediated cellular responses may be important in the context of pulmonary fibrosis.

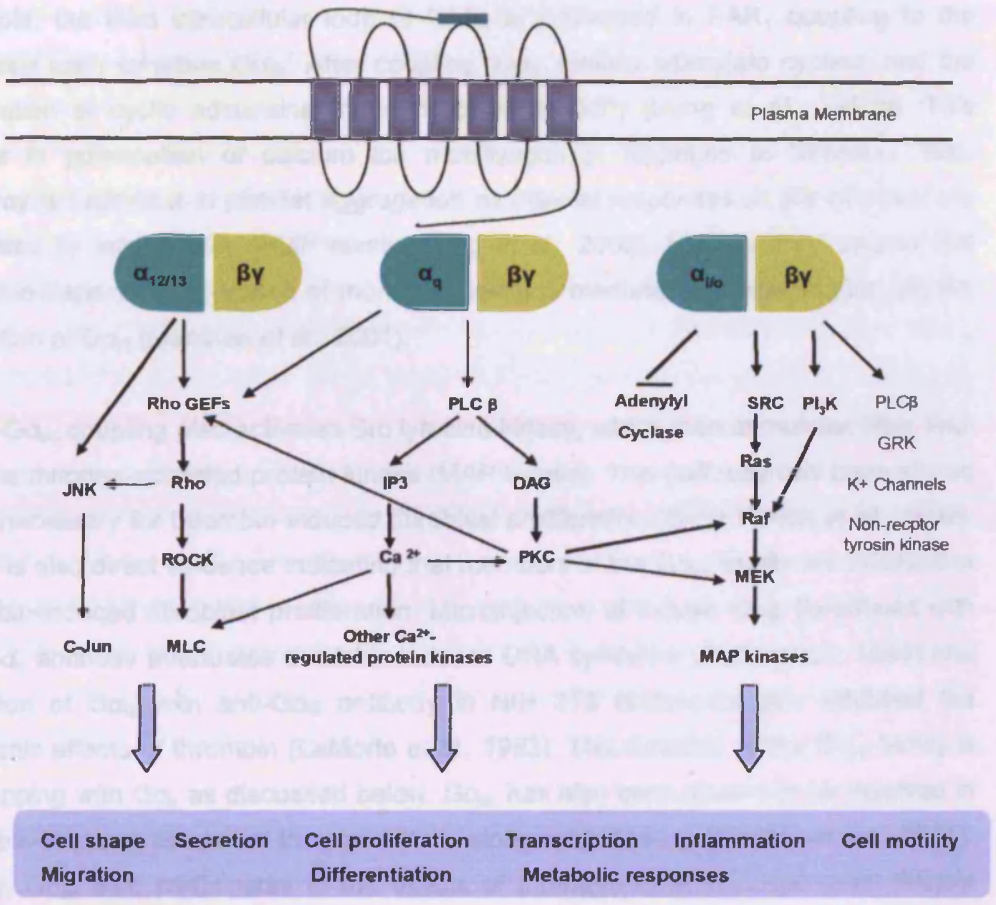


Figure 1.4 Major PAR_1-G-protein mediated signalling pathways

Figure shows the major G protein-mediated signalling pathways after coupling to PAR_1. In general, the $G\alpha_{12/13}$ pathway couples to Rho guanine-nucleotide exchange factors (GEFs) resulting in activation of Rho, Rho kinase (ROCK), and myosin light chain (MLC) kinase; $G\alpha_q$ activates phospholipase C-β (PLCβ) to generate inositol triphosphate (IP3) and diacylglycerol (DAG), which mobilize intracellular Ca^{2+} and activate protein kinase C (PKC); whereas the $G\alpha_i$ pathway inhibits adenylyl cyclase. The Gβγ subunits activate pathways including phosphoinositide 3-kinase (PI3k), PLCβ, G protein-coupled receptor kinases (GRK), K^+ channels, and non-receptor tyrosin kinases. (Figure modified from (Coughlin, 2000; McLaughlin *et al.*, 2005)

PAR_1-$G\alpha_{i/o}$ pathway

$G\alpha_{i/o}$ family members are generally pertussis-toxin sensitive, apart from $G\alpha_z$. Different binding sites are required for PAR_1 coupling to distinct G protein α subunits. For example, the third intracellular loop of PAR_1 is implicated in PAR_1 coupling to the pertussis-toxin sensitive $G\alpha_{i/o}$. After coupling, $G\alpha_{i/o}$ inhibits adenylate cyclase and the generation of cyclic adenosine monophosphate (cAMP) (Hung *et al.*, 1992a). This results in potentiation of calcium ion mobilisation in response to thrombin. $G\alpha_{i/o}$ pathway is important in platelet aggregation as platelet responses at site of injury are regulated by intracellular cAMP levels (Yang *et al.*, 2002). Studies have shown that thrombin-dependent activation of murine platelets is mediated, at least in part, via the activation of $G\alpha_{i2}$ ((Jantzen *et al.*, 2001).

PAR_1-$G\alpha_{i/o}$ coupling also activates Src tyrosine kinase, which then stimulates Ras, Raf, and the mitogen-activated protein kinase (MAP kinase). This pathway has been shown to be necessary for thrombin-induced fibroblast proliferation (Della Rocca *et al.*, 1999). There is also direct evidence indicating that members of the $G\alpha_{i/o}$ family are involved in thrombin-induced fibroblast proliferation. Microinjection of mouse lung fibroblasts with anti-$G\alpha_o$ antibody attenuates thrombin-induced DNA synthesis (Baffy *et al.*, 1994) and inhibition of $G\alpha_{i2}$ with anti-$G\alpha_{i2}$ antibody in NIH 3T3 fibroblasts also inhibited the mitogenic effects of thrombin (LaMorte *et al.*, 1993). This function of the $G\alpha_{i/o}$ family is overlapping with $G\alpha_q$ as discussed below. $G\alpha_{i/o}$ has also been shown to be involved in thrombin-induced fibroblast transformation along with $G\alpha_{12/13}$ (Martin *et al.*, 2001). Finally, $G\alpha_{i/o}$ also participates in the effects of thrombin in endothelial cells. Recent studies demonstrated that PAR_1-$G\alpha_{i/o}$ coupling is responsible for the increased expression of thrombospondin-1 (THBS-1) induced by thrombin in vascular endothelial cells (McLaughlin *et al.*, 2005).

PAR_1-$G\alpha_{q/11}$ pathway

PAR_1-$G\alpha_{q/11}$ is another major thrombin signaling pathway. The cononical effector molecules of activated $G\alpha_q$ are the β-isoforms of phospholipase C (PLC-β) (Babich *et al.*, 1990). $G\alpha_q$ binds and stimulates PLC-β to initiate inositol lipid signalling. PLC-β triggers phosphoinositide hydrolysis (PIP_2) to generate inositol triphosphate (IP_3) and diacylglycerol (DAG). This in turn mobilizes calcium from intracellular stores, increasing the intracellular calcium concentration from approximately 10^{-7}M to 10^{-3}M, and also causes the activation of protein kinase C (PKC) (Berridge, 1993). Calcium, as a second messenger, activates calcium-dependent kinases and phosphatases such as CaM kinases, Ras guanine-nucleotide exchange factors (GEFs) and MAP kinases. PKC is a

key enzyme in ERK (one of the MAP kinases) signaling and Raf is the primary target of PKC (Kolch, 2000).

There are 5 members in the $G\alpha_{q/11}$ family, but $G\alpha_q$ and $G\alpha_{11}$ are the most ubiquitously distributed across tissues and they share the most structural and functional similarities (Hubbard and Hepler, 2006). Genetic studies using whole animal models demonstrate the importance of $G\alpha_q$ family members in cardiac, lung, brain and platelet function among other physiological processes (Hubbard and Hepler, 2006). In vivo studies with $G\alpha_q$-deficient mice also point to a role for $G\alpha_q$ in the regulation of airway smooth muscle contraction/relaxation (Borchers *et al.*, 2003). $G\alpha_q$-deficient mice display defective lung function (assessed by both tracheal tension and in vivo lung function measurement) and the mice fail to respond to allergen challenge. $G\alpha_q$ is the only member of the $G\alpha_q$ family that is expressed in platelets, and $G\alpha_q$-deficient mice have shown increased bleeding times and impaired platelet function such as aggregation in response to collagen (Offermanns *et al.*, 1997). Moreover, platelets derived from $G\alpha_q$-deficient mice are completely unresponsive to thrombin stimulation, suggesting the unique role of $G\alpha_q$ in mediating the effects of thrombin in platelets (Moers *et al.*, 2004). This is consistent with *in vitro* studies showing that PAR_1 activation by thrombin leads to calcium-dependent shape changes of human platelets and this is achieved by the activation of $G\alpha_q$ (Hung *et al.*, 1992b).

In vitro studies have also shown an important role for $G\alpha_q$ signalling in mediating thrombin's effects on fibroblasts and endothelial cells (Dery *et al.*, 1998). As previously described (Section **1.7.1.4**), thrombin exerts important effects on fibroblasts and promotes their proliferation and differentiation. The mitogenic effects of thrombin in fibroblasts are mediated by PAR_1 coupling to $G\alpha_q$, since inhibition of $G\alpha_q$ by microinjection of anti-$G\alpha_q$ antibodies caused a dramatic decrease of fibroblast DNA synthesis after thrombin stimulation (LaMorte *et al.*, 1993; Baffy *et al.*, 1994). This is accompanied by a decrease in calcium mobilization, suggesting that $G\alpha_q$ also modulates thrombin-induced calcium mobilization in fibroblasts. PAR_1-$G\alpha_q$ is required for fibroblast differentiation since thrombin does not induce the differentiation in fibroblasts derived from $G\alpha_q$-deficient mice (Marinissen *et al.*, 2003).

In endothelial cells, PAR_1-$G\alpha_q$ is responsible for NFκB activation and ICAM-1 transcription induced by thrombin and this is coordinately regulated by $G\beta\gamma$ dimers dissociated from $G\alpha_q$ (Rahman and MacNee, 1998; Miho *et al.*, 2005). Thrombin-induced calcium mobilization in endothelial cells is also $G\alpha_q$-dependent as

demonstrated by two individual studies (McLaughlin *et al.*, 2005; Singh *et al.*, 2007). However, conflicting results have been shown by these two studies in terms of thrombin-induced endothelial permeability and the discrepancy may be explained by the methodologies used in both studies. Data obtained by McLaughlin and colleagues suggest that $G\alpha_{12/13}$ rather than $G\alpha_q$ is responsible for thrombin-induced endothelial permeability. This conclusion is based on experiments showing that the Rho kinase inhibitor Y-27632 rather than the calcium chelator BAPTA-AM inhibits this effect, but is not based on direct experimental evidence from $G\alpha_{12/13}$ or $G\alpha_q$ inhibition. As classically, Rho kinase and calcium are downstream effectors of $G\alpha_{12/13}$ and $G\alpha_q$, respectively, $G\alpha_{12/13}$ rather than $G\alpha_q$ was felt to mediate thrombin-induced endothelial permeability. In contrast, Singh and colleagues reported that $G\alpha_q$ is responsible for mediating thrombin-induced endothelial cell permeability, and this was confirmed by using $G\alpha_q$ mutants that block $G\alpha_q$ signalling directly. This study also demonstrated that Rho rather than Ca^{2+} is involved as a downstream effector of $G\alpha_q$ in mediating this response of thrombin.

PAR_1-$G_{12/13}$ pathway

PAR_1 also transduces signals via the α subunits of $G\alpha_{12}$ and $G\alpha_{13}$ (Offermanns *et al.*, 1994). Upon activation, $G\alpha_{12}$ and $G\alpha_{13}$ appear to selectively interact with the small Rho GTPase, Rho (which will be discussed in the forthcoming section). As discussed above, $G\alpha_q$ deficiency results in decreased airway smooth muscle contraction, $G\alpha_{12}$ and $G\alpha_{13}$ signalling pathway is also shown to be responsible for the hyperresponsiveness of airway smooth muscle contraction in rats after repeated antigen challenge (Chiba and Misawa, 2001). The levels of $G\alpha_{12}$ and $G\alpha_{13}$ proteins are found to be upregulated in bronchial smooth muscle after 24 hours repeated antigen challenge, and this is associated with a dramatic increase in bronchial smooth muscle contraction.

As already mentioned, activation of PAR_1 by thrombin induces fibroblast transformation via both $G\alpha_{i/o}$ and $G\alpha_{12/13}$. This study further demonstrated the involvement of downstream serum response factor (SRF) and NFκB in this response (Martin *et al.*, 2001). There is also evidence that PAR_1 coupling to $G\alpha_{12/13}$ mediates thrombin-induced fibroblast transformation via the activation of the Jun N-terminal kinase (JNK) pathway (Marinissen *et al.*, 2003). Apart from mediating mitogenic effects of thrombin on fibroblasts, the PAR_1-$G\alpha_{13}$ pathway is found to be responsible for thrombin-stimulated DNA synthesis and cell migration in smooth muscle cells (Seasholtz *et al.*, 1999). As mentioned previously, PAR_1 coupling to $G\alpha_q$ mediates platelet aggregation in a calcium-dependent manner (Hung *et al.*, 1992b). Studies have also shown that

activation of PAR_1 can induce platelet aggregation via a calcium-independent mechanism mediated by $G\alpha_{12/13}$ (Offermanns *et al.*, 1994). The role of $G\alpha_{13}$ in platelets has been demonstrated in studies by Moers and colleagues showing that platelets lacking $G\alpha_{13}$ fail to respond to thrombin (Moers *et al.*, 2004). The $G\alpha_{13}$ signalling cascade includes the activation of Rho and thus induction of cytoskeletal changes affecting cell migration. In endothelial cells, thrombin-induced endothelial barrier dysfunction is mediated by the PAR_1-$G\alpha_{12/13}$ pathway (Birukova *et al.*, 2004a). However, studies by Singh and colleagues have demonstrated that the PAR_1-$G\alpha_q$ pathway mediates thrombin-induced endothelial barrier dysfunction. The discrepancy in findings may be explained by the different concentrations of thrombin used in both studies, since the concentration of thrombin (50 nM) in studies by Birukova and colleagues is 5 times higher than that used in studies by Singh and colleagues. It is well-recognized that thrombin at higher concentrations signals via different mechanism compared to thrombin at lower concentrations.

PAR_1-$G\beta\gamma$ pathway

GPCR-G protein signalling is no longer regarded as a linear pathway from agonist-activated receptor to Gα-stimulated effector. Gβγ dimers also function as signalling molecules (Hall *et al.*, 1999). For example, Gβγ has been found to mediate airway SMC proliferation induced by thrombin. After activation by thrombin, Gβγ is dissociated from the Gα subunit and mediates SMC growth via the activation of PI3-kinase (Krymskaya *et al.*, 1999). Furthermore, the involvement of Gβγ in PAR_1 signalling has been established by studies showing that Gβγ regulates thrombin-induced NFκB activation and ICAM-1 expression in endothelial cells (Rahman *et al.*, 1999; Rahman *et al.*, 2001; Rahman *et al.*, 2002). Gβγ signalling may also participate in apoptosis by activation of the PI3K-Akt pathway, a major pathway involved in regulating apoptosis (Jun *et al.*, 2003). In Chinese hamster embryonic fibroblasts, the sequestration of Gβγ dimers from $G\alpha_{i2}$ and $G\alpha_q$ inhibits thrombin-regulated PI3K-Akt pathway (Goel *et al.*, 2004). Moreover, Gβγ dimers from $G\alpha_o$ were found to be necessary for thrombin-induced changes in endothelial barrier permeability (Vanhauwe *et al.*, 2002).

Functional selectivity of PAR_1-G protein signalling

It is important to note that functional selectivity does exist as a feature of PAR_1-G protein interactions when PAR_1 is activated by different agonists. For example, both thrombin and the PAR_1 specific agonist peptide, TFLLR, activate PAR_1 on endothelial cells to influence barrier permeability and calcium mobilization. However, PAR_1 activated by thrombin preferentially couples to $G\alpha_{12/13}$ and therefore produces a greater

effect on barrier permeability, whereas activation by TFLLR is associated with more efficient receptor coupling to $G\alpha_q$ and a greater effect on calcium mobilization (McLaughlin *et al.*, 2005). This phenomenon has been termed 'functional selectivity', and may provide a potential explanation for the distinct gene expression profiles observed in cytokine-stimulated endothelial cells in response to activation of PAR_1 by APC and thrombin (Riewald and Ruf, 2005).

1.7.4 Role of signal molecules downstream of PAR_1-G protein coupling in pulmonary fibrosis

The substantial network of signalling pathways downstream of PAR_1-G protein coupling requires multiple interactions at several levels by important protein kinases as shown in figure 1.4. These protein kinases actually mediate most of the signal transduction in eukaryotic cells and they are activated by phosphorylation. Of particular interest, the roles of PKC isoforms, mitogen-activated protein kinases (MAPKs) and RhoA/Rho kinase downstream of PAR_1-G protein coupling in pulmonary fibrosis will be discussed in detail in the forthcoming sections.

1.7.4.1 Protein kinase C

1.7.4.1.1 Overview

The intracellular serine/threonine kinase, PKC, is ubiquitously expressed in various cell types. Twelve different isozymes have been identified to date that differ in their structure, biochemical properties, tissue distribution, subcellular localization, and substrate specificity. The PKC isozymes are generally classified as conventional (α, β1, β2, γ), novel (δ, ε, η, θ, μ), atypical (ζ, λ), and isoform v which is currently not classified (Hayashi *et al.*, 1999; Dempsey *et al.*, 2000). These isozymes can be further divided into two groups: Ca^{2+}-dependent (conventional) PKC, and Ca^{2+}-independent (novel and atypical isozymes) PKC which do not require Ca^{2+} for their activation.

All PKC isozymes can be activated by diacylglycerol (DAG) apart from ζ and λ. DAG is produced following the activation of PLC. Activation of membrane receptor by stimuli results in activation of PLC or phospholipase A2 (PLA_2) via a G-protein-dependent mechanism. Activated PLC then hydrolyzes PIP_2 to produce DAG and IP_3. The production of IP_3 leads to the release of endogenous Ca^{2+}, which in turn binds to the cytosolic PKC and translocates PKC to the membrane. The PKC finally interacts with DAG and is transformed into a fully active enzyme (Mochly-Rosen and Gordon, 1998).

Individual PKC isozymes have been shown to mediate many cellular responses, namely, permeability, contraction, migration, hypertrophy, proliferation, apoptosis, and secretion. These cellular responses have been implicated in clinical disorders such as pulmonary edema, adult respiratory distress syndrome, interstitial lung disease, asthma, and pulmonary hypertension (Dempsey *et al.*, 2000; Dempsey *et al.*, 2007).

The important roles of PKCs in the lung have been demonstrated by studies involving isolated organ preparations and whole animal models, including knockout and transgenic mice (Littler *et al.*, 2003; Littler *et al.*, 2005). Persistent hypoxic pulmonary vasoconstriction leads to vascular remodeling and fixed pulmonary hypertension, and these effects are abolished by PKC-ε deletion (Littler *et al.*, 2003). However, there are few PKC-related studies in the settings of the human lung. Gene expression profiling of lung tissue from patients with pulmonary fibrosis failed to demonstrate an upregulation of genes encoding the PKC isozymes (Zuo *et al.*, 2002). However, much of the regulation of PKC occurs at the protein level, including phosphorylation and localization, gene array studies may therefore underestimate the importance of PKCs in various disease settings, including pulmonary fibrosis.

1.7.4.1.2 Protein kinase C signalling

The PKC-dependent signal transduction pathways have been found to regulate many intracellular events in fibroblasts involved in the development of fibrosis. Previous studies have shown that at least four PKCs are expressed in interstitial fibroblasts including PKC-α, PKC-γ, PKC-δ and PKC-ε (Ludwicka-Bradley *et al.*, 2000; Luzina *et al.*, 2006). Fibroblast proliferation and differentiation are critical for the development of fibrosis, and current evidence suggests that these events are mediated by different PKC isozymes after thrombin stimulation *in vitro*. For example, inhibition of PKC-α, but not PKC-ε, by antisense oligonucleotides prevents thrombin-induced lung fibroblast proliferation, whereas thrombin-induced myofibroblast differentiation is mediated by PKC-ε (Bogatkevich *et al.*, 2001; Bogatkevich *et al.*, 2003; Bogatkevich *et al.*, 2005). Resistance to apoptosis in lung fibroblasts is another important mechanism contributing to the pathogenesis of pulmonary fibrosis and can also be induced by thrombin. This process is found to be mediated by PKC-ε, not PKC-α following PAR_1 activation (Bogatkevich *et al.*, 2005).

Collagen contraction is important in tissue remodeling in fibrotic lung disease. The ability of fibroblasts to contract three-dimensional collagen gels has therefore been used as an *in vitro* model of the tissue contraction which characterises both normal

repair and fibrosis. Studies have shown that thrombin can induce collagen gel contraction mediated by human lung fibroblasts and inhibition of both PKC-ε and PKC-δ reduce this effect of thrombin, suggesting that both PKC-ε and PKC-δ may contribute to tissue remodeling in these lung diseases (Fang *et al.*, 2004; Fang *et al.*, 2008). Fibroblasts not only play important roles in fibrosis via their ability to synthesize extracellular matrix, but also via the release of a host of cytokines and chemokines. Studies have demonstrated that PKC-γ is responsible for thrombin-induced synthesis of IL-8 by lung fibroblasts (Ludwicka-Bradley *et al.*, 2000). IL-8 may play a key role in the pathogenesis of pulmonary fibrosis by recruiting and activating neutrophils within the local microenvironment (Miller *et al.*, 1992).

PKC may also contribute to pulmonary fibrosis by regulating the endothelial barrier. It is reported that PKC-α plays an important role in lung endothelial injury in response to thrombin activation of PAR_1 and it is via the downstream Rho kinase pathway (reviewed in (Siflinger-Birnboim and Johnson, 2003)). In contrast, studies have also shown that thrombin can promote endothelial permeability via the activation of PKC-ζ via a Rho kinase-independent mechanism (Li *et al.*, 2004). In addition, thrombin has been found to induce ICAM-1 and VCAM-1 expression via the activation of PKC-δ in endothelial cells (Wu and Aird, 2005; Bijli *et al.*, 2008). Finally, studies have shown that thrombin induces IL-8 production in lung epithelial cells via the activation of PKC-α.

In conclusion, current evidence suggests that PKC-α, PKC-γ, PKC-δ and PKC-ε are likely to play important roles in the pathogenesis of pulmonary fibrosis.

1.7.4.2 Mitogen-activated protein kinases pathways

As described above, PAR_1-G protein signaling pathways can ultimately lead to the activation of the MAPKs family (please see figure 1.4 and figure 1.5). The MAP kinase signal transduction pathways are the major pathways by which extracellular stimuli are transmitted as intracellular signals. They are key components of intracellular signaling pathways that control important cellular activities, such as gene expression, cell proliferation and programmed cell death. The elucidation of the molecular mechanisms whereby GPCRs activate MAPKs is believed to be central to understanding how these receptors regulate these important cellular events. MAP kinases are a group of highly conserved, eukaryote-specific serine/threonine kinases. There are three major subgroups in this family, namely c-Jun amino-terminal kinases (JNK1/2/3), extracellular signal-regulated kinases (ERK1/2), and p38 kinases (p38α, β, γ, δ). In general, the MAPKs are sequentially phosphorylated by upstream kinases (Figure 1.5) (Kyriakis

and Avruch, 2001). Briefly, the MAPK cascade is initiated by the MAPK kinase kinases (MAPKKKs or MEKKs). The MEKKs activate the downstream MAPK kinases (MAPKKs or MEKs) by phosphorylation of specific serine and threonine amino acid residues (Widmann *et al.*, 1999). MEKs in turn phosphorylate certain threonine and tyrosine residues of MAPKs. Upon activation, the MAPKs translocate to the cell nucleus where they phosphorylate nuclear proteins, including transcription factors and histone proteins, thereby affecting an intricate balance of regulatory molecules controlling gene expression (Treisman, 1996).

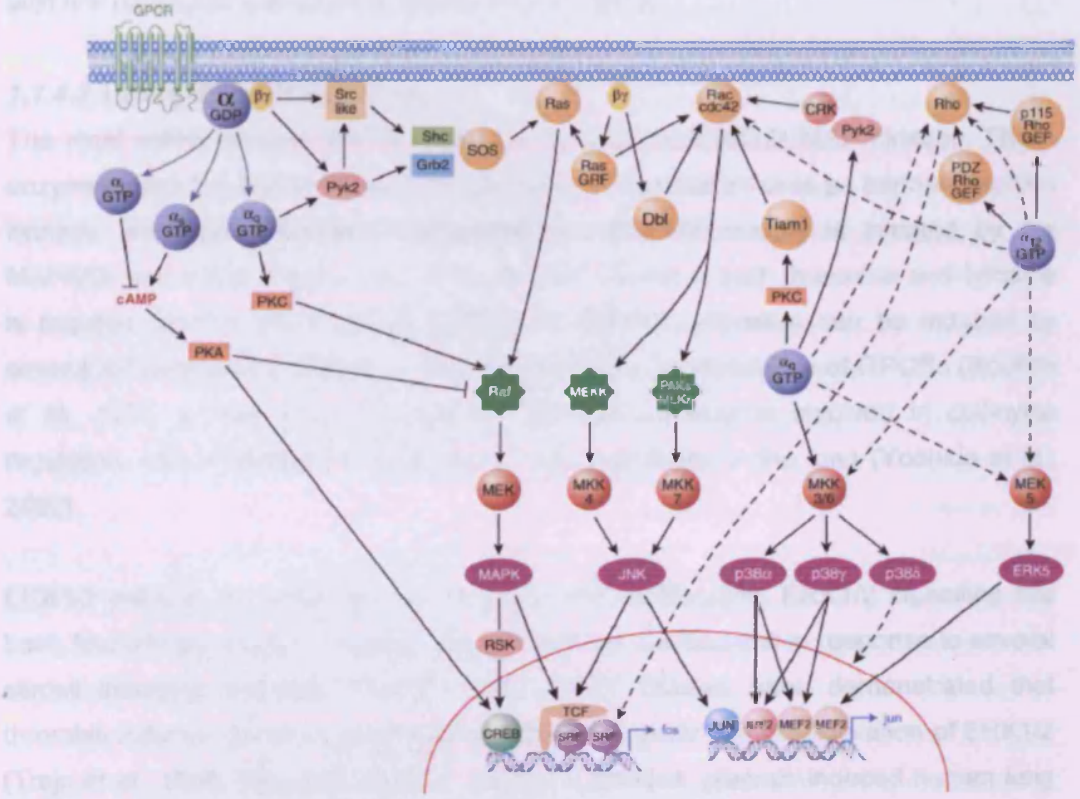


Figure 1.5 Multiple signaling pathways link G protein-coupled receptors to the nucleus. Molecules linking G protein-coupled receptors to MAPK, JNK, p38 and ERK5 are depicted. Arrows, positive stimulation; blocked lines, inhibition; dashed lines, interactions not well established. Adapted from Gutkind SJ etal [online] http://www.stke.org/cgi/content/full/OC_sigtrans;2000/40/rel 2000

The MAP kinase family has been the subject of intense investigation for many years owing to their major role in cellular growth, differentiation and survival, and the pathological effects (e.g. carcinogenesis) that result from dis-regulated MAPK activity. MAPKs represent an important point of convergence for several different signalling pathways; they affect multiple aspects of normal airway function and also significantly contribute to lung pathophysiology such as inflammation and the survival of resident lung cells (Yoshida *et al.*, 2002; Pelaia *et al.*, 2005; Bennett, 2006). The important involvement of MAPKs in lung fibrosis has been suggested by studies demonstrating that activated MAPKs are significantly increased in lung homogenates from patients with IPF compared with controls (Yoshida *et al.*, 2002).

1.7.4.2.1 PAR_1-ERK1/2 signalling

The most widely studied MAPK cascade is that of the ERK1/2 MAP kinases. These enzymes were the first MAPKs to be identified in mammalian cells as serine/threonine kinases. The phosphorylation cascade in the ERK1/2 module is initiated by the MAPKKK and c-Raf (Figure 1.5). Dual phosphorylation of both threonine and tyrosine is required for the full activation of ERK1/2. ERK1/2 activation can be induced by several different growth factors, cytokines, as well as by stimulation of GPCRs (Boulton *et al.*, 1991; Johnson and Lapadat, 2002). This pathway is involved in cell-cycle regulation, cell proliferation and protection from apoptosis in the lung (Yoshida *et al.*, 2002).

ERK1/2 pathway is preferentially involved in cell proliferation. ERK1/2 signalling has been found to be critical in mediating lung fibroblast proliferation in response to several stimuli including thrombin (Pages *et al.*, 1993). Studies have demonstrated that thrombin induces fibroblast proliferation and differentiation via the activation of ERK1/2 (Trejo *et al.*, 1996; Bogatkevich *et al.*, 2005). In addition, plasmin-induced human lung fibroblast proliferation is mediated by PAR_1 activation and downstream ERK1/2 pathway (Mandal *et al.*, 2005). Moreover, studies have demonstrated that the ERK1/2 pathway plays an important role in PAR_1-activation-induced SMC proliferation (Lee *et al.*, 2001; Schauwienold *et al.*, 2003).

ERK1/2 may also be important in pulmonary fibrosis as an anti-apoptotic effector for fibroblasts. In vitro studies have demonstrated that thrombin inhibits lung fibroblast apoptosis via the activation of ERK1/2 pathway (Chalmers *et al.*, 2003). In lung tissues from patients with IPF, the activation of ERK1/2 has been found to be significantly decreased in epithelial cells but not fibroblasts, suggesting that the persistent

accumulation of fibroblasts in the lung is partially caused by decreased apoptosis mediated by ERK1/2 signalling (Yoshida *et al.*, 2002). Finally, ERK1/2 pathway is involved in pulmonary fibrosis by mediating inflammatory chemokine and growth factor release. Studies have shown that PAR_1-activation-induced IL-8 production in airway epithelial cells requires the activation of both ERK1/2 and JNK (Ostrowska and Reiser, 2008b). PDGF is mitogenic to fibroblasts and ERK1/2 also mediates thrombin-induced PDGF production in endothelial cells (Wu and Aird, 2005).

1.7.4.2.2 PAR_1-JNK signalling

JNKs are activated upon exposure of cells to cytokines, growth factors, and environmental stress, such as UV irradiation or heat shock (Davis, 2000). Dual threonine and tyrosine phosphorylation of JNK by MAKK4 and MAKK7, results in JNK activation and nuclear location. JNK signalling has been implicated in multiple biological processes, including embryonic development, inflammation, cell proliferation and differentiation, and apoptosis regulation (Bennett, 2006).

JNKs may be involved in pulmonary fibrosis through its regulation of apoptosis. The role of JNK in regulating apoptosis is complicated since it may exhibit pro- or anti-apoptotic effects, which are depend on the strength, duration and type of stimulus (Lin, 2003; Liu and Lin, 2005). Evidence for the pro-apoptotic effect of JNK in the lung has been provided in studies using cigarette-smoke-induced lung apoptosis model in rats (Kuo *et al.*, 2005). In addition, activated JNK is found to be increased in epithelial and endothelial cells but not fibroblasts in lung sections from patients with IPF, which may explain the excessive epithelial cell apoptosis and fibroblast apoptotic resistance in pulmonary fibrosis (Yoshida *et al.*, 2002). There is also *in vitro* evidence that JNK is pro-apoptotic in the lung. Studies have shown that leukocyte elastase induces apoptosis of human lung epithelial cells by a mechanism involving the activation of PAR_1 and downstream JNK pathway (Suzuki *et al.*, 2005). Taken together, these studies suggest that JNK is pro-apoptotic, which differs from the antiapoptotic effect of ERK1/2 in pulmonary fibrosis.

Studies have demonstrated that activation of JNK is involved in thrombin-induced fibroblast transformation (Marinissen *et al.*, 2003). The JNK pathway has also been found to mediate PAR_1 activation-induced inflammatory mediator release, including IL-8 in airway epithelial cells (Ostrowska and Reiser, 2008b), ICAM-1 in endothelial cells (Miho *et al.*, 2005), and tissue factor in endothelial cells (Wu and Aird, 2005).

1.7.4.2.3 PAR_1-P38 signalling

P38 is a ubiquitous and highly conserved, proline-directed serine/threonine protein kinase. It was initially discovered in a pharmacological screen when a cellular functional assay was used for the identification of compounds that modulate TNF-α production from a lipopolysaccharide (LPS)-stimulated human monocytic cell line (Lee *et al.*, 1994). The primary role of p38 is considered to be in mediating inflammatory response in disease settings (Schindler *et al.*, 2007). It is reported that p38 is associated with pulmonary events and is predominately involved in inflammation in pulmonary disease (Konstan and Davis, 2002; de Boer *et al.*, 2007). This is supported by studies showing that the production and action of many of the potential mediators of airway inflammation are dependent on the p38 kinase cascade (Pelaia *et al.*, 2005; Schindler *et al.*, 2007).

Studies so far have demonstrated that P38 is mainly involved in PAR_1-induced inflammatory mediator release in pulmonary fibrosis. For example, thrombin has been reported to induce CCL2 production via the activation of p38 MAPK in both human endothelial cells (Marin *et al.*, 2001) and smooth muscle cells (Brandes *et al.*, 2001). P38 is also found to mediate thrombin-induced tissue factor expression in human pulmonary artery endothelial cells (Wu and Aird, 2005). However, this result contrasts with studies showing that JNK activation mediates thrombin-induced tissue factor expression in endothelial cells (Steffel *et al.*, 2006). The discrepancy may be explained by the different thrombin concentrations used in both studies. As already mentioned previously, thrombin at different concentrations may signal via distinct pathways. In addition, the p38 pathway has also been shown to mediate thrombin-induced tissue factor production in smooth muscle cells (BelAiba *et al.*, 2006). Moreover, thrombin-induced VCAM-1 expression in endothelial cells is mediated by the p38 pathway (Steffel *et al.*, 2006). Finally, p38 may be important in pulmonary fibrosis by regulating endothelial cell permeability. Studies have shown that thrombin-induced endothelial cell permeability is p38 MAPK-dependent (Borbiev *et al.*, 2004).

1.7.4.3 RhoA/Rho kinase

Low molecular weight G proteins of the Rho subfamily belong to the RAS superfamily which consists of Rho, Rac and Cdc42. There are three members in the Rho family, namely RhoA, RhoB and RhoC. RhoA is the best characterized member and will be discussed in detail in this thesis. Unlike heterotrimeric G proteins, the Rho GTPases are not directly activated through ligand binding to GPCRs. Activation of small Rho GTPases and their downstream effectors is mediated through heterotrimeric G proteins

and primarily through G proteins of the $G\alpha_{12/13}$ family (Sah *et al.*, 2000). However, recent evidence strongly indicates that the Rho family can also be activated by the $G\alpha_{q/11}$ family (reviewed in (Hubbard and Hepler, 2006)).

The activation of Rho GTPases is similar as the process of G protein activation. The cycling of Rho GTPases between the inactive GDP-bound form and the activated GTP-bound form is controlled by guanosine nucleotide exchange factors (GEFs), GTPase-activating proteins (GAP) and guanine nucleotide dissociation inhibitors (GDIs). GEFs promote exchange of GTP for GDP and thereby activate Rho; GAPs inactivate Rho by enhancing the intrinsic GTP-hydrolysis activity; GDIs bind to the GDP-bound Rho protein and allow translocation between membranes and the cytosol.

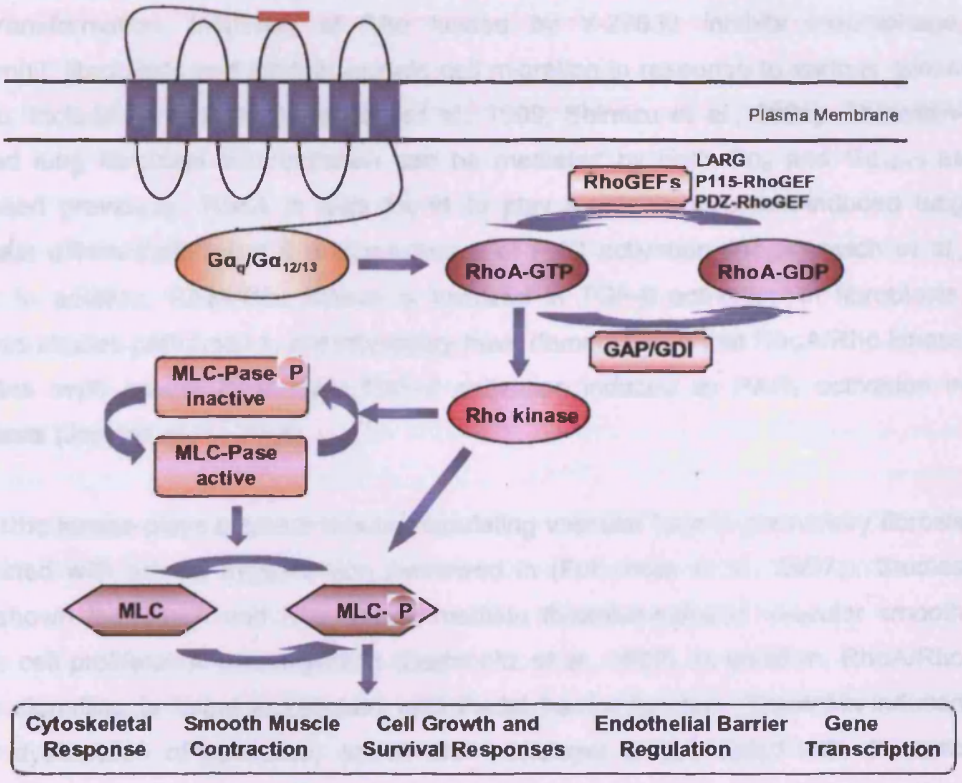


Figure 1.6 Schematic illustrations of RhoA/Rho kinase and downstream effector MLC activation. Both $G\alpha_q$ and $G\alpha_{12/13}$ can activate RhoA, which in turn leads to the activation of Rho kinase. Following activation, Rho kinase activates MLC directly. Rho kinase can also activate MLC indirectly by phosphorylating the MLC phosphatases. GEF: guanine nucleotide exchange factors; GAP: GTPase activating proteins; GDI: guanine nucleotide dissociation inhibitors; MLC: myosin light chain

RhoA is involved in the control of the organization of actin cytoskeleton, cell migration, smooth muscle contraction, gene transcription and cell growth (Sah *et al.*, 2000; Wennerberg and Der, 2004; Wennerberg *et al.*, 2005). Rho kinase (ROCK) is a major target of RhoA, including ROCKα and ROCK β (Kaibuchi *et al.*, 1999). Myosin light chain (MLC) is one of the major downstream substrates of RhoA/Rho kinase signalling (Amano *et al.*, 1996). Rho kinase can activate MLC directly via phosphorylation mechanism. It can also indirectly activate MLC by phosphorylating the myosin-binding subunit of MLC phosphatase, which in turn renders the phosphatase inactive and thus prevents MLC dephosphorylation (Kimura *et al.*, 1996).

There is a wealth of *in vitro* evidence that RhoA/Rho kinase signalling mediates several cellular events related to pulmonary fibrosis, including cell migration, cell survival and cell transformation. Inhibition of Rho kinase by Y-27632 inhibits macrophage, neutrophil, fibroblasts and smooth muscle cell migration in response to various stimuli in vitro, including thrombin (Seasholtz *et al.*, 1999; Shimizu *et al.*, 2001). Thrombin-induced lung fibroblast differentiation can be mediated by both $G\alpha_q$ and $G\alpha_{12/13}$ as discussed previously. RhoA is also found to play a role in thrombin-induced lung fibroblast differentiation and it is downstream of PKC activation (Bogatkevich *et al.*, 2003). In addition, RhoA/Rho kinase is involved in TGF-β activation in fibroblasts. Previous studies performed in our laboratory have demonstrated that RhoA/Rho kinase mediates αvβ6 integrin-dependent TGF-β activation induced by PAR_1 activation in fibroblasts (Jenkins *et al.*, 2006).

RhoA/Rho kinase plays a critical role in modulating vascular tone in pulmonary fibrosis associated with arterial hypertension (reviewed in (Fukumoto *et al.*, 2007)). Studies have shown that RhoA and Rho kinase mediate thrombin-induced vascular smooth muscle cell proliferation and migration (Seasholtz *et al.*, 1999). In addition, RhoA/Rho kinase signalling is found to regulate endothelial barrier function. Thrombin-induced barrier dysfunction of pulmonary endothelial monolayer is associated with dramatic cytoskeletal reorganization, activation of actomyosin contraction, and gap formation. RhoA/Rho kinase activation is a key mechanism of this effect of thrombin as inhibition of RhoA and Rho-kinase by dominant-negative constructs significantly blocked thrombin-induced endothelial barrier dysfunction (Birukova *et al.*, 2004b). Moreover, RhoA/Rho kinase regulates thrombin-induced ICAM-1 expression in endothelial cells (Anwar *et al.*, 2004). Finally, Rho kinase has been shown to be involved in irreversible platelet aggregation induced by PAR_1 activation (Missy *et al.*, 2001).

1.7.5 The development of PAR_1 antagonists

As already alluded to, increasing evidence has been accumulating regarding the deleterious role of PAR_1 in lung injury and fibrosis. Therefore, blocking PAR_1 or interfering with the signalling pathways downstream of PAR_1 activation may be a promising therapeutic strategy. This section will briefly summarize recent progress in the development of PAR_1 antagonists for both clinical use and experimental purposes.

1.7.5.1 Tethered ligand-based PAR_1 antagonists

A number of peptide and peptidomimetic PAR_1 antagonists aiming for clinical use are currently available, but the clinical utility of these agents remains to be established. For example, the heterocycle-based peptide-mimetic of PAR_1 antagonists, indole-based RWJ-56110 and indazole-based RWJ-58259 have been reported to be selective for PAR_1 *in vitro* and have been proved to be effective in non-human primate models of thrombosis, including a cynomolgus monkey arterial injury model (Derian *et al.*, 2003; Maryanoff *et al.*, 2003). Studies with another related indole-based PAR_1 antagonist showed protective effects in a rat model of liver fibrosis at a dose of 1.5 mg kg^{-1} day^{-1}. In contrast, the non-peptide PAR_1 antagonists such as the pyrroloquinazoline analogues, SCH 79797, has been reported to be toxic for lung fibroblasts (Sokolova and Reiser, 2007) and have potential off-target effects (Di Serio *et al.*, 2007).

Recently, a new series of PAR_1 antagonists have been developed. These antagonists are based on the core structure of the tetracyclic piperidine alkaloid, himbacine, from Australian magnolia trees (Chackalamannil *et al.*, 2005). The most potent one among these antagonists demonstrates good affinity with a K_i of 4.3 nM against PAR_1, up to 70% blockade of platelet aggregation in the cynomolgus monkey model at a dose of 3 mg kg^{-1}, and approximately 60% oral bioavailability (Chelliah *et al.*, 2007).

The orally active PAR_1 antagonist, SCH-205831, developed by Schering, which inhibits PAR_1 by competitively inhibiting the tethered ligand-binding site has been found to be effective as an antithrombotic agent in humans (Chackalamannil *et al.*, 2005). A phase II clinical trial of a related PAR_1 antagonist, SCH 530348, has been reported to be successful, providing hope for the availability of an effective orally available PAR_1-targeted antithrombotic compound (Camerer, 2007) (http//:www.sch-plough.com/Schering_plough/news/ release.jsp?releaseID=977603).

1.7.5.2 PAR_1 antagonists that blocks PAR_1-dependent activation of G proteins

As mentioned in previous sections, PAR_1 mediates its cellular responses via different G proteins. An entirely different strategy for targeting PAR_1 is to specifically inhibit PAR_1 interactions with individual G proteins and there are currently several promising tools available, such as pepducins (Leger *et al.*, 2006), G-protein selective PAR_1 antagonists (Caden Biosciences) and G protein minigenes encoding the COOH-terminal peptide sequences of various Gα subunits (Gilchrist *et al.*, 2001). These latter tools are available to further our understanding in experimental models *in vitro* and possibly *in vivo*, but are unlikely to progress as potential therapeutic agents for use in humans.

Pepducins are designed to bind to the receptor-G protein interface on the inner leaflet of the plasma membrane and have been studied in the context of PAR_1 and PAR_4 signalling (Covic *et al.*, 2002b). For example, the anti-PAR_1 pepducin, P1pal-12, has been shown to effectively inhibit PAR_1-induced platelet aggregation (Covic *et al.*, 2002b). However, the specificity of pepducins is still controversial. Studies by Stampfuss and colleagues have argued that the anti-PAR_4 pepducin, P4pal-10, may also inhibit other GPCR-mediated responses, including thromboxane receptors (Stampfuss et al 2003 9 1447). In addition, the cross-inhibition between PAR_1 and PAR_4 by pepducins has been reported (Covic *et al.*, 2002a). Therefore, pepducins may be a potential tool for predicting an interaction between PAR_1 and PAR_4, rather than specific inhibitors for inhibiting PAR_1-G protein interaction (reviewed in (Leger *et al.*, 2006).

It has become increasingly clear that the carboxyl termini of the G protein α subunits are critical in G-protein signalling (Reviewed in (Gilchrist *et al.*, 1998; Wess, 1998)). Dominant negative constructs targeting the carboxyl terminus can be used as competitive inhibitors of receptor-G protein interactions. In this regard, Dr Gilchrist and co-workers have generated minigene plasmid constructs that encode the 11 unique COOH-terminal sequences of various Gα subunits (Gilchrist *et al.*, 2001). Instead of competing with binding of Mg^{2+}, these G protein minigenes directly interfere with G protein-receptor interaction. The C-terminal region of various G protein α subunits has been shown to be not only the essential region for receptor contact, but also critical in determining the specificity of GPCR-G protein interactions (Hamm and Gilchrist, 1996; Hamm, 1998). In addition, substituting a single amino acid has been shown to annul the ability of the $G\alpha_i$ to bind the A1 adenosine receptor, further supporting the unique importance of the C-terminal region for receptor-G protein signalling (Gilchrist *et al.*, 1998). These minigenes have previously been shown to effectively inhibit G-protein

signalling, including thrombin-mediated cellular effects (Ellis *et al.*, 1999; Gilchrist *et al.*, 2001; Vanhauwe *et al.*, 2002).

Similar as the Gα minigenes, novel G protein selective PAR_1 antagonists have also been designed according to the 11 amino acids found at the carboxyl-terminus of Gα subunits. Q94 is a recently developed novel PAR_1 selective $G\alpha_q$ protein signalling antagonist (Caden Biosciences) and has been used in this thesis. The $G\alpha_q$ antagonist Q94, is a small cell permeable molecule (MW<500) that meets the Lipinski Rule of Five. The compound was chosen on the basis of its ability to compete for binding at the carboxyl-terminus of PAR_1, with a high affinity receptor binding peptide probe (HABP) designed around the 11 amino acids found at the carboxyl-terminus of $G\alpha_q$. The HABP probe (T2-14) was selected from a proprietary library of $G\alpha_q$ related peptides, constructed by sequential substitution of the 11 native C-terminal amino acids with each of the 20 known amino acids. IC_{50} values for binding of the HABPs to the receptor carboxyl-terminus showed that T2-14 has a higher binding affinity for PAR_1 than the native $G\alpha_q$ C-terminal peptide (Appendix, Table 1). The selective nature of the T2-14 for PAR_1 was further supported by the observations that both a β2AR-x probe (identified by screening the activated β2-adrenergic receptor with a $G\alpha_s$ library), and RHO8 probe (identified by screening activated Rhodopsin with a $G\alpha_t$ library) showed little or no binding affinity for PAR_1. Q94 was found to compete off the highly selective T2-14, from activated PAR_1 receptors with an IC_{50} of 916 nM (Appendix, Figure A.1, left panel). Additionally, this compound was found to effectively inhibit TRAP induced calcium transients (Appendix, Figure A.2, right panel).

Taken together, inhibition of PAR_1 seems to be safer than direct inhibition of the activating proteinases as it allows selective inhibition of the potentially deleterious receptor-mediated cellular effects of coagulation proteinases, while preserving their essential roles in homeostasis. As a G protein-coupled receptor, selective inhibition of PAR_1-G protein pathways may then represent a novel therapeutic approach for a number of respiratory conditions associated with excessive coagulation proteinase signalling.

1.8 CCL2 (CC chemokine ligand 2)

1.8.1 Overview

The aim of this thesis is to examine the expression of CCL2 in lung fibroblasts following PAR_1 activation by thrombin, and to examine the signalling pathways involved. CCL2, also known as JE in the mouse, is a member of the CC chemokine family. Chemokines

are small (7-10 kDα) secreted proteins that function in leukocyte trafficking and recruitment, and are characterised as basic heparin-binding proteins (Puneet *et al.*, 2005). They are distinguished from other cytokines by being the only members of the cytokine family that act on G protein-coupled receptors. The chemokine families are named according to the structure of conserved cysteine-containing motifs. In the CXC chemokine family, the first two NH2-terminal cysteines are separated by a non-conserved amino acid residue. The CC chemokines are defined by the two cysteine residues being adjacent to each other (Chung, 2001). The C chemokines only contain one cysteine amino acid.

Currently four monocyte chemoattractant proteins (MCPs) have been characterized in humans, of which CCL2 (MCP-1) was the first to be discovered and the best characterized. CCL12 (MCP-5) is only found in mouse and it has recently been suggested that CCL12 is the functional homologue of human CCL2 (Sarafi *et al.*, 1997; Moore *et al.*, 2006). CC chemokine receptor 2 (CCR2) is the major high affinity CCL2 signalling receptor. Two isoforms of CCR2 have been cloned and termed CCR2A and CCR2B (Charo *et al.*, 1994). CCR2 also acts as a receptor for three other MCPs, including CCL8 (MCP-2), CCL7 (MCP-3) and CCL13 (MCP-4), which can bind to multiple chemokine receptors as indicated in figure 1.7 (Keane *et al.*, 2005)

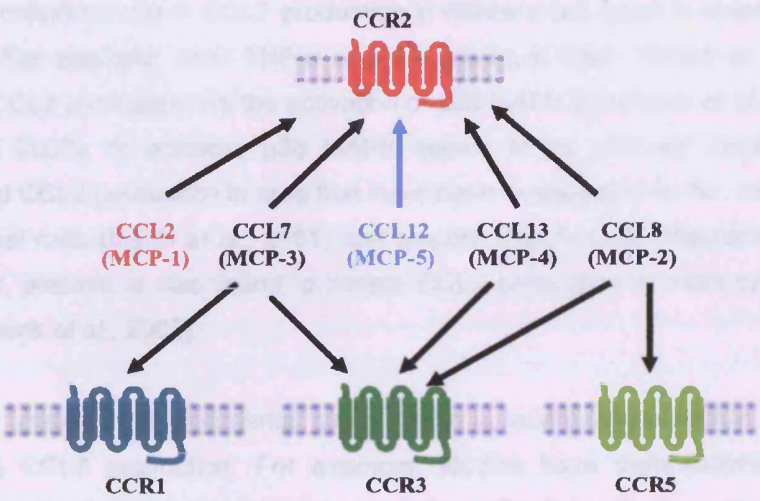


Figure 1.7 Multiple CCR2 specific ligands. CCL12 is the functional homologue of human CCL2 and only found in the mouse.

1.8.2 Regulation of CCL2 production

1.8.2.1 Mediators of CCL2 production

CCL2 is produced by several cell types, including endothelial cells, epithelial cells, mononuclear cells, fibroblasts, osteoblasts, keratinocytes, astrocytes and mast cells (Puneet *et al.*, 2005). This chemokine is produced in response to a variety of inflammatory signals, including early response cytokines, PDGF, IL-1, IL-4, IL-6, IL-10, IL-31, TNF-α, IFN-γ, TGF-β and bacterial LPS (reviewed in (Rose *et al.*, 2003; Ip *et al.*, 2007). It is also produced in response to thrombin (Colotta *et al.*, 1994), FXa (Bachli *et al.*, 2003) and viral infection (Bussfeld *et al.*, 2000). Additionally, CCL2 expression can be induced by agents and factors that stress cells (Shyy *et al.*, 1995; Glabinski *et al.*, 1996; Faller *et al.*, 1997). In contrast, CCL2 production can be inhibited by some agents that suppress inflammation, such as retinoic acid, glucocorticoids, dexamethasone (Zhou *et al.*, 2007), and estrogen (Frazier-Jessen and Kovacs, 1995).

1.8.2.2 Mitogen-activated protein kinase signalling and CCL2 production

The intracellular signal transduction pathways involved in CCL2 production have not been fully elucidated. However, MAPKs have been demonstrated to play a role in regulating CCL2 production in response to a variety of stimuli, including thrombin. Within the MAPK family, the p38 MAPK has been particularly associated with inflammatory cytokine production. Indeed, a variety of studies have suggested that p38 MAPK plays an important role in CCL2 production in different cell types in response to various stimuli. For example, both TNF-α and CXCL2 have been shown to induce endothelial cell CCL2 production via the activation of p38 MAPK (Goebeler *et al.*, 1999; Calderon *et al.*, 2006). In addition, p38 MAPK seems to be uniquely involved in thrombin-induced CCL2 production in cells that have been investigated so far, including human endothelial cells (Marin *et al.*, 2001) and smooth muscle cells (Brandes *et al.*, 2001). Moreover, plasmin is also found to induce CCL2 production in monocytes via p38 MAPK (Burysek *et al.*, 2002).

Although JNK is related to environmental stress, there is recent evidence that JNK is also involved in CCL2 production. For example, studies have demonstrated that pneumocystis stimulates CCL2 production by alveolar epithelial cells through a JNK-dependent mechanism as inhibition of JNK inhibited this response, while inhibition of both p38 and ERK1/2 had no effect (Wang *et al.*, 2007). In addition, JNK has also been found to mediate IL-18-induced CCL2 production in rheumatoid arthritis synovial tissue fibroblasts (Amin *et al.*, 2007). In contrast, the third MAPK family member, ERK1/2, which is associated with growth and differentiation signals, has not been found to

mediate CCL2 production so far. In addition to MAPKs, recent studies have examined the role of PKC in CCL2 production, and demonstrated that PKCα but not PKCδ is involved in IL-18-induced CCL2 production by rheumatoid arthritis synovial tissue fibroblasts via the activation of JNK (Amin *et al.*, 2007).

1.8.2.3 CCL2 gene regulation

Several functional binding sites for transcription factors are present in the 5'-flanking region of the human CCL2 gene, including NF-κB, Sp1, AP-1, TRE, and NF-IL-6 (Ping *et al.*, 1996; Ping *et al.*, 1999a; Ping *et al.*, 1999b; Finzer *et al.*, 2000; Ping *et al.*, 2000). Among these transcription factors, NF-κB has been shown to be important and two closely located NF-κB binding sites, termed A1 and A2, were identified in the 5'-flanking region of the human CCL2 gene (Ueda *et al.*, 1994; Ueda *et al.*, 1997). Among the variety of inducers of CCL2 expression, TNF-α is perhaps the most potent and regulation of CCL2 by TNF-α occurs primarily at the transcription level. It is reported that NF-κB plays a critical role in TNF-α-induced CCL2 gene expression in epithelial cells as deletion of the two NF-κB binding sites completely abolished this effect of TNF-α (Xing and Remick, 2007). In addition, NF-κB is also found to be involved in TNF-α-induced CCL2 gene expression in primary human endothelial cells and murine fibroblasts (Ping *et al.*, 1996; Goebeler *et al.*, 2001). It has also been reported that both SP1 and NF-κB are important in TNF-α-induced CCL2 expression (Ping *et al.*, 1999b; Ping *et al.*, 2000; Guo *et al.*, 2003). In contrast, other studies have suggested that AP-1 is necessary for TNF-α-mediated induction of murine CCL2 gene in clonal osteoblastic and HPV 18-positive carcinoma cells (Ping *et al.*, 1996; Finzer *et al.*, 2000). This discrepancy in findings may be explained by differences in the cell type examined.

In addition, NF-κB is also an important transcriptional factor for LPS and IL-1β-induced CCL2 gene expression as suggested by several studies (Ueda *et al.*, 1997; Xing and Remick, 2007). In conclusion, NF-κB, SP 1 and AP-1 appear to be involved in CCL2 gene expression with NF-κB the most important transcriptional factor identified in terms of CCL2 induction in response to inflammatory stimuli.

1.8.3 Role of CCL2 in pulmonary fibrosis

CCL2 is a potent chemoattractant for mononuclear cells, basophils, T cells, immature dendritic cells, and natural killer cells (Rose *et al.*, 2003). Although other chemokines have similar functions and the unique CCL2 receptor CCR2 acts as receptor for other ligands, CCL2 has been shown to be essential for monocyte recruitment in several inflammatory models *in vivo* and in fibroproliferative lung disease, such as ARDS and

IPF (Lu *et al.*, 1998; Rose *et al.*, 2003). In addition, a recent study has reported an elevation of CCL2 protein levels in BALF from patients with mustard-gas-induced pulmonary fibrosis (Emad and Emad, 2007).

In the setting of human IPF, several studies have demonstrated the elevation of CCL2 mRNA and protein levels in pulmonary epithelial cells, monocytes/macrophages, vascular endothelial and in smooth muscle cells by *in situ* hybridisation and immunohistochemistry (Antoniades *et al.*, 1992; Standiford *et al.*, 1993). Moreover, unpublished studies from the host laboratory have shown that fibroblast-like cells in the IPF lung are strongly immunoreactive for CCL2 (Figure 1.8) (Mercer *et al*, unpublished). In addition, pulmonary fibroblasts from IPF patients exhibit a reduced ability to downregulate CCL2 expression in the presence of either PGE_2 or the glucocorticoid, dexamethasone (Standiford *et al.*, 1993). Cultured primary human lung fibroblasts derived from fibrotic lesions from subjects with IPF display a greater ability to express CCL2 in the presence of either TNF-α or IL-1β, as compared to normal controls (Standiford *et al.*, 1993). These findings strongly support the notion that CCL2 may play an important role in the pathogenesis of pulmonary fibrosis.

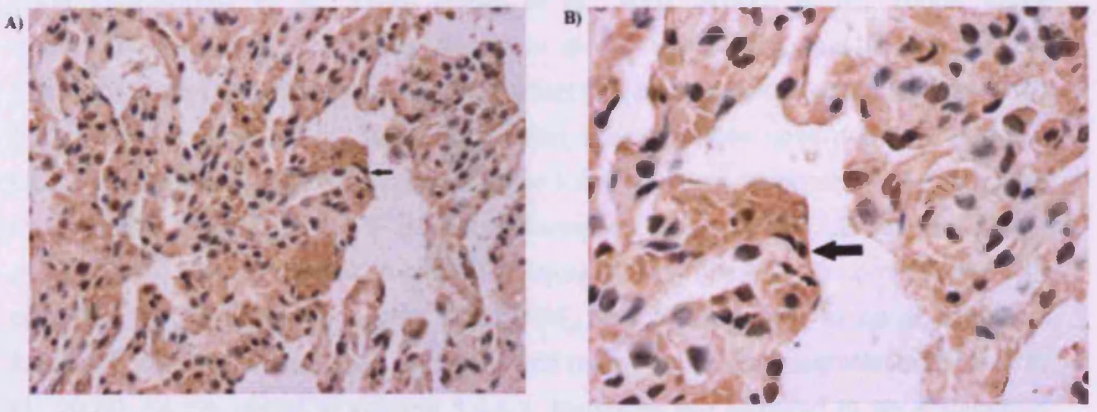


Figure 1.8 Immunohistochemical localisation of CCL2 in a lung tissue specimen from a patient with IPF. The spindle-shaped (fibroblast-like) cells are positive for CCL2 by immunostaining (Panel A and B, as indicated by arrows). These cells are negative for the epithelial maker CK7 and the macrophage marker CD68. Magnification (x 100 and x 400). (Image kindly provided by Dr Paul Mercer, Centre for Respiratory Research, UCL)

There is compelling evidence that CCL2 plays a pathological role as a pro-inflammatory mediator following lung injury. CCL2 is upregulated in response to bleomycin challenge in rodents in a time-dependent manner (Smith *et al.*, 1996; Zhang

et al., 2004). CCL2 mRNA levels are significantly elevated in BALF cells at 24 hours post-bleomycin challenge, while in lung tissue, it is maximally elevated at 7 days, correlating with eosinophil and mononuclear cell infiltration. In addition to its ability to modulate inflammatory cell recruitment in the lung, CCL2 has been suggested to be involved in Th2 cytokine polarization in the context of pulmonary fibrosis (Hogaboam *et al.*, 1998a; Gu *et al.*, 2000). Karpus and co-workers have shown that T cell activation in the presence of CCL2 enhances IL-4 generation (Karpus *et al.*, 1997). Neutralization of CCL2 leads to a reduction in IL-4 expression levels and an augmentation in IFN-γ production by CD4+ lymphocytes when co-cultured with fibroblasts (Hogaboam *et al.*, 1998a). Lymph node cells from CCL2-deficient mice synthesize extremely low levels of IL-4, IL-5 and IL-10, but normal amounts of INF-γ and IL-2, and CCL2-deficient mice are unable to mount a type 2 response, which is thought to be important in driving lung fibrosis in animal models (Gu *et al.*, 2000). Taken together, CCL2 may have both a direct role in the pathogenesis of pulmonary fibrosis through its effects on inflammatory cell infiltration, and an indirect role by controlling of T helper cell polarization.

Furthermore, CCL2 also exerts pro-fibrotic effects. It stimulates fibroblast collagen production via the induction of TGF-β_1 and a reduction in PGE_2 production, and CCL2 may also contribute to excessive collagen production via the recruitment of fibrocytes (Gharaee-Kermani *et al.*, 1996; Moore *et al.*, 2003; Moore *et al.*, 2005). CCL2 treatment of rat lung fibroblasts results in the concentration- and time-dependent expression of type I procollagen, and this effect can be attenuated with antisense TGF-β_1 (Gharaee-Kermani *et al.*, 1996). In addition, recent studies have demonstrated that CCL2 upregulates α-SMA and procollagen I and III gene expression in fibroblasts derived from the lungs of IPF patients (Murray *et al.*, 2008). CCL2 can limit PGE_2 production in alveolar epithelial cells after injury, thus promoting fibroproliferation and collagen deposition by fibroblasts since PGE_2 has been shown to be able to limit fibroblast proliferation, collagen synthesis, and promote collagen degradation (Moore *et al.*, 2003). As discussed in **section 1.4.1.2**, fibrocytes are believed to be a source of fibroblasts and therefore contribute to lung fibrosis. Studies have demonstrated that CCR2, the high-affinity receptor for CCL2, mediates fibrocyte recruitment via the interaction with CCL2 (Moore *et al* 2005). This study has also shown that CCL2 induces collagen synthesis by fibrocytes. Moreover, deficiency of CCR2 has also been shown to be protective in both fluorescein isothiocyanate (FITC) and bleomycin-induced pulmonary fibrosis (Moore *et al.*, 2001; Gharaee-Kermani *et al.*, 2003). Finally, overexpression of a CCL2 dominant-negative inhibitor causes a consistent reduction in the degree of bleomycin-induced fibrosis (Inoshima *et al.*, 2004).

In addition, a very recent study from Liu and colleagues proposed that CCL2 may contribute to fibrosis via its ability to regulate apoptosis (Liu *et al.*, 2007). Apoptosis of lung structural cells is crucial in the process of normal tissue repair and insufficient apoptosis of lung fibroblasts may contribute to the development of fibrosis. In this study, CCL2 was added to fibroblast cultures and was found to inhibit staurosporin- and serum deprivation-induced apoptosis. Interestingly, CCL2 mediates fibroblast apoptotic resistance via the release of IL-6 since IL-6 release is upregulated after CCL2 stimulation, and neutralization of IL-6 by specific antibodies block CCL2-induced inhibition of fibroblast apoptosis. Taken together, these findings suggest that CCL2 may be an important mediator in fibrotic lung disease by promoting a range of cellular responses.

1.9 Summary, hypothesis and aims

Pulmonary fibrosis is the end stage of a heterogeneous group of disorders, characterised by excess deposition of extracellular matrix within the pulmonary interstitium. Excessive local procoagulant activity is a characteristic feature of pulmonary fibrosis and likely to be important in its pathogenesis. In this chapter, the role of the high affinity thrombin receptor PAR_1 in promoting pro-inflammatory and pro-fibrotic cellular effects of thrombin has been reviewed. As PAR_1 is a G-protein-coupled receptor, PAR_1-G protein signal transduction and downstream signalling pathways in pulmonary fibrosis were also been reviewed.

Previous studies from our laboratory have shown that the protection from bleomycin-induced lung inflammation and fibrosis is associated with a reduction in the upregulation of the PAR_1-inducible mediator, CCL2 (Howell *et al.*, 2005). However, the signalling pathway leading to CCL2 production via PAR_1 remains unknown. The existing evidence for CCL2 as an important pro-inflammatory and pro-fibrotic mediator in pulmonary fibrosis makes it a compelling candidate for PAR_1-G protein signalling studies. The aim of this thesis is to elucidate the G-protein signal transduction pathways by which activation of PAR_1 leads to CCL2 production in order to identify potential novel targets for therapeutic intervention.

This thesis will therefore specifically address the following hypothesis:
PAR_1 activation leads to the production and release of the pro-inflammatory and pro-fibrotic chemokine, CCL2, via its interaction with heterotrimeric G-proteins and activation of multiple downstream signalling cascades.

To address this hypothesis, my project has the following specific aims:

I) To elucidate the specific G-protein signal transduction pathway by which activation of PAR_1 induces the production of CCL2 by mouse lung fibroblasts *in vitro.*

II) To examine the effect of PAR_1 selective G-protein signalling antagonists on mouse lung fibroblast CCL2 expression *in vitro.*

III) To delineate the signal transduction pathways downstream of PAR_1-G-protein coupling for CCL2 production induced by thrombin in mouse lung fibroblasts *in vitro.*

IV) To examine the role of PAR_1 in thrombin-induced CCL2 production in primary human lung fibroblasts.

Chapter 2: Materials & Methods

Materials

2.1. General plastic ware, chemicals and solvents

All chemicals were of analytical grade or above and obtained from *Sigma Aldrich* (UK), unless otherwise indicated. All water used for the preparation of buffers was distilled and deionised using a Millipore Water Purification System (Millipore R010 followed by Milli-Q Plus; Millipore Ltd, Watford, UK). Cell culture plastic-ware was purchased from NUNC (NUCN™, Denmark). Polypropylene centrifuge tubes and pipettes were obtained from Falcon (New Jersey, USA), unless otherwise stated. Other disposable plasticware was purchased from Sterilin (Ashford, Middlesex, UK).

2.2 Reagents

2.2.1 Cell culture reagents

High-glucose Dulbecco's Modified Eagle's Medium (DMEM), penicillin-streptomycin, L-glutamine, 0.25% trypsin-EDTA, geneticin and hygromycin were from Gibco BRL (Gibco BRL, Paisley, UK). Foetal calf serum (FCS) was obtained from Invitrogen (Invitrogen, UK). New born calf serum (NCS) (heat inactivated) was purchased from Imperial Laboratories (Andover, Hampshire, UK). Luria Bertani (LB) broth for bacterial culture was purchased from Invitrogen (Invitrogen, UK).

2.2.2 Kits

Wizard® Plus Minipreps DNA Purification system (Promega, UK); Qiagen® Plasmid Maxi Kit (Qiagen, UK); OptEIA™ Mouse CCL2 ELISA Set, TMB Substrate Reagents set Microtest™ 96-well ELISA plates were from BD Biosciences, Pharmingen (San Diego, CA, USA). Lipofectamine 2000 was purchased from Invitrogen (Invitrogen, UK). BCA™ protein Assay Reagents A & B were purchased from Pierce (Pierce, USA). Enhanced chemiluminescence (ECL) kit was purchased from Amersham (Buckinghamshire, UK). DNA-free™ kit was obtained from Ambion (Ambion Ltd. UK). RT-PCR kit for cDNA synthesis was purchased from Applied Biosystems (Roche, Lewes, UK). **Others:** Proteinase inhibitor cocktail was obtained from Sigma (Sigma, UK). TRIzol was purchased from Gibco (Gibco BRL, Paisley, UK).

2.3 Coagulation factors, cytokines and peptides

Thrombin, extracted from human plasma, was purchased from Calbiochem (Merck Biosciences, UK). The PAR_1 agonist peptide TFLLR-NH_2, the reverse peptide RLLFT-

NH_2 and FTLLR-NH_2 were synthesized and provided by Professor R. Mecham (University of Washington, MO, USA). The PAR_1 agonist peptide SFLLRN was purchased from Sigma (Sigma, UK). Purified human factor Xa (FXa) was obtained from American Diagnostica Inc. (Greenwich, CT, USA). The PAR_2 agonist peptide SLIGKV was purchased from *Auspep* (Australia). The PAR_4 agonist peptide AYPGKF was obtained from *Bachem* (Bachem, Germany), and the reverse peptide YAPGKF was purchased from *Chempep* (Chempep, USA). TNF-α was purchased from *R&D Systems* (UK). All preparations were dissolved in DMEM culture medium, aliquot and stored at -80 °C.

2.4 Inhibitors

PAR_1 antagonist RWJ-58259 was kind gift from Dr Claudia Derian (Johnson & Johnson Pharmaceutical Research & development, USA). The novel small molecule PAR_1 antagonist Q94 which selectively blocks the PAR_1 interaction with $G\alpha_q$ was developed by Dr Annette Gilchrist (Caden Biosciences, USA). Inhibitors used are listed in Table 2.1. RWJ-58259 was added to the cells 15 minutes before subsequent experiments. Q94 was added to the cells 3 hours prior to the addition of treatments, and cycloheximide was put onto the cells 1 hour prior to the subsequent experiments. All the other inhibitors used were added 30 minutes before subsequent treatment. All the inhibitors were dissolved in appropriate solutions as indicated in Table 2.1, aliquot and stored at -80 °C.

Anti-PAR_1 antibodies ATAP2 and WEDE15 were employed in this study to block PAR_1 cleavage by thrombin. ATAP2 is a monoclonal IgG_1-anti PAR_1 antibody which binds to an epitope within the PAR_1 tethered ligand domain, and was purchased from *Santa Cruz* (Sc-13503, Santa Cruz Biotechnology, CA, USA). WEDE15 is also a monoclonal IgG_1-anti PAR_1 antibody that binds to the hirudin-like domain, and was obtained from *Beckman Coulter* (Beckman Coulter, UK). Both antibodies were dissolved in double-distilled water, ATAP2 stock was kept at fridge and WEDE15 stock was stored at -20 °C. Antibodies were added to the cells (final concentration: 25 μg/ml of each) 30 minutes prior to the subsequent treatments with thrombin.

All inhibitors and antagonists used in this thesis are listed in **Table 2.1**.

Inhibitors	Function	Dissolved by	Stock concentration	Source
Actinomycin D	RNA inhibitor	Ethanol	10 µg/ml	Sigma
BAPTA-AM	Calcium chelator	DMSO	20 mM	Calbiochem
Blebbestatin	MLC inhibitor	DMSO	20 mM	Calbiochem
C3 exoenzyme	RhoA inhibitor	ddH_2O	10 µg/ml	Tebu-Bio
c-Raf kinase inhibitor	c-Raf kinase inhibitor	DMSO	10 mM	Calbiochem
Cycloheximide	Protein synthesis inhibitor	ddH_2O	10 µg/ml	Sigma
GF109203X	Broad-spectrum PKC inhibitor	DMSO	10 mM	Calbiochem
Gö6976	Ca^{2+}-dependent PKC inhibitor	DMSO	10 mM	Calbiochem
H-1152	Rho kinase inhibitor	ddH_2O	10 mM	Calbiochem
PD98059	MEK inhibitor	DMSO	50 mM	Calbiochem
Pertussis toxin	$G\alpha_{i/o}$ inhibitor	ddH_2O	50 mg/ml	Calbiochem
Q94	$G\alpha_q$ inhibitor	DMSO	10 mM	Caden Biosciences
Ro-318425	Broad-spectrum PKC inhibitor	DMSO	10 mM	Calbiochem
RWJ-58259	PAR_1 antagonist	DMSO	10 mM	Johnson & Johnson
SB 203580	P38 inhibitor	DMSO	10 mM	Calbiochem
SB 202474	Inactive control for SB203580	DMSO	10 mM	Calbiochem
TI-JIP	JNK inhibitor	DMSO	10 mM	Calbiochem
U73122	PLC inhibitor	DMSO	20 mM	Calbiochem
U73343	Inactive control for U73122	DMSO	20 mM	Calbiochem
U0126	MEK inhibitor	DMSO	10 mM	Calbiochem
Y-27632	Rho kinase inhibitor	ddH_2O	30 mM	Calbiochem

Table 2.1 Inhibitors and antagonists

2.5 Antibodies.

Antibodies used in this study are listed in Table 2.2.

Antibody Name	Antibody Property	Supplier and Catalogue N°	Dilution Used	Application
Anti-p38	Rabbit polyclonal IgG	Cell signalling Technology, MA, USA # 9212	1:2500	Western Blot
Anti-phospho-p38	Rabbit polyclonal IgG	Cell signalling Technology, MA, USA # 9215	1:1000	Western Blot
Anti ERK1/2 IgG	Rabbit polyclonal IgG	Cell Signalling Technology, MA, USA # 9102	1:5000	Western Blot
Anti phospho-ERK1/2 (Thr202/Tyr 204) IgG	Rabbit polyclonal IgG	Cell Signalling Technology, MA, USA # 9101	1:5000	Western Blot
Anti c-Raf IgG	Rabbit polyclonal IgG, 400 µg/ml	Cell Signalling Technology, MA, USA # 9422	1:5000	Western Blot
Anti phospho-c-Raf (Ser289/296/301) IgG	Rabbit polyclonal IgG	Cell Signalling Technology, MA, USA # 9431	1:5000	Western Blot
Anti MLC IgG	Rabbit polyclonal IgG	Cell Signalling Technology, MA, USA # 3672	1:1000	Western Blot
Anti-phospho-MLC IgG	Rabbit polyclonal IgG	Kind gift from Dr James M. Staddon (Eisai London Research Laboratories, UK)	1:500	Western Blot
Anti rabbit IgG-HRP	Goat polyclonal IgG, 400 µg/ml	Santa Cruz Biotechnology, CA, USA sc-2004	1:25000	Western Blot
Anti-human CCL2 (capture)	Mouse IgG_{2B}	R&D, # MAB 679	2 µg/ml	ELISA
Biotinylated anti-human CCL2 (detecting)	Goat IgG	R&D, # BAF 279	2 µg/ml	ELISA
Anti-CCL2	Goat IgG	R&D, # AF-279-NA	10 µg/ml	Immunocytofl -uorecence

Table 2.2 List of antibodies for ELISA, Western Blot and Immunocytofluorecence.

2.6 Primers

Primers used for gene expression studies are listed in Table 2.2. Primers were designed by Dr Chris Scotton (Centre for Respiratory Research, UCL).

Primers	Forward	Reverse
mCCL2	AGCTCTCTCTTCCTCCACCAC	CGTTAACTGCATCTGGCTGA
$mPAR_1$	AGCCAGCCAGAATCAGGAG	AGGGGGACCAGTTCAAATGTA
$mPAR_2$	CGGGACGCAACAACAGTAA	GTTCCACCGGAACCCCTTTC
$mPAR_3$	TGCCAAAGTGGCATAAATGT	TCCAGCCCTCTATGTCAGAA
$mPAR_4$	AGACCCCCAGCCATCTACGA	GTCTGAGGACTTCGGCTCCT
hPR_1	CCATCGTTGTGTTCATCCTG	GACCCAAACTGCCAATCACT
$hPAR_2$	CACCATCCAAGGAACCAATAG	TGCAGAAAACTCATCCACAGA
$hPAR_3$	TCAGAGTGGCATGGAAAATG	AAGGCAGAAAAGGGGAACTC
$hPAR_4$	GAAGGCTGTACTGGGTCGAA	AAGTGACCTCCGCTAGTGACA

Table 2.3 Primers used for RT-PCR and quantitative real time RT-PCR
H=human, M=murine

2.7 *Vectors.*

2.7.1 MEK1-pBabePuro constructs

The MEK1-pBabePuro constructs were kind gifts from Dr. Christopher J Marshall (Cancer Research in UK): wild type MEK1-pBabePuro contains the wild type rat MEK1 gene; the dominant negative MEK1-pBabePuro construct transcribes inactive MEK1 encoding substitution of Alanine for Serine at Ser-221; constitutively active MEK1-pBabePuro construct transcribes constitutively active MEK1encoding Glutamic Acid substitutions at Ser-217 and Ser-221 (Cowley et al 1994). The wild type MEK1 sequence was obtained from Genebank (Rat MKK, Genbank #: Z30163). The mutants were directionally subcloned into the pBabePuro vector using the BamHI and EcoRI restriction sites in the MCS region (See Figure 2.1).

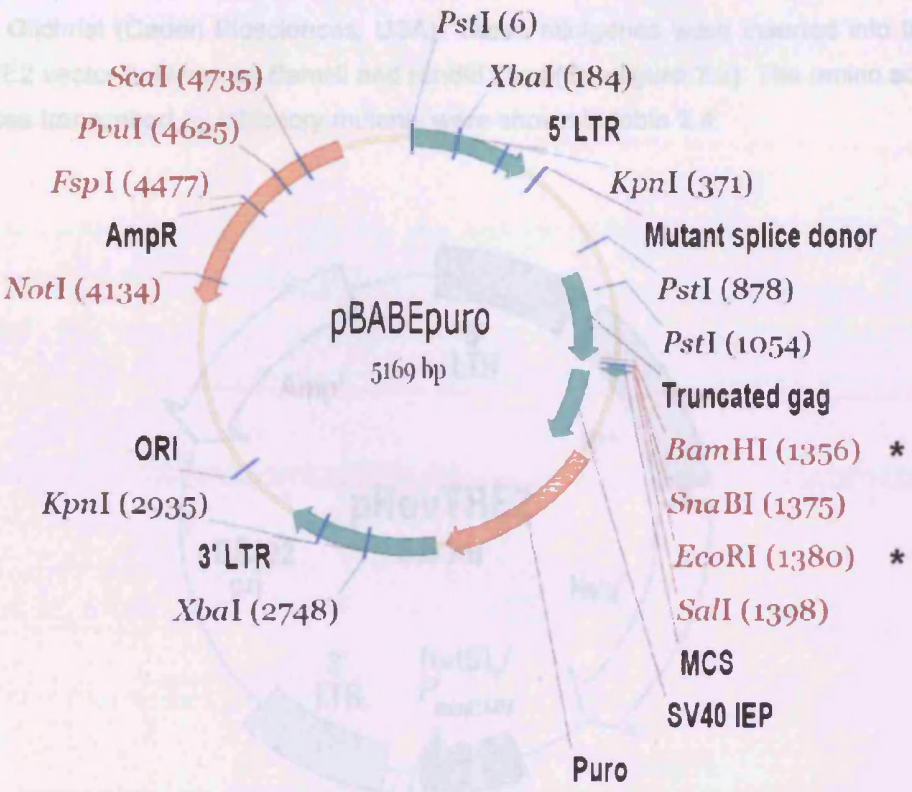
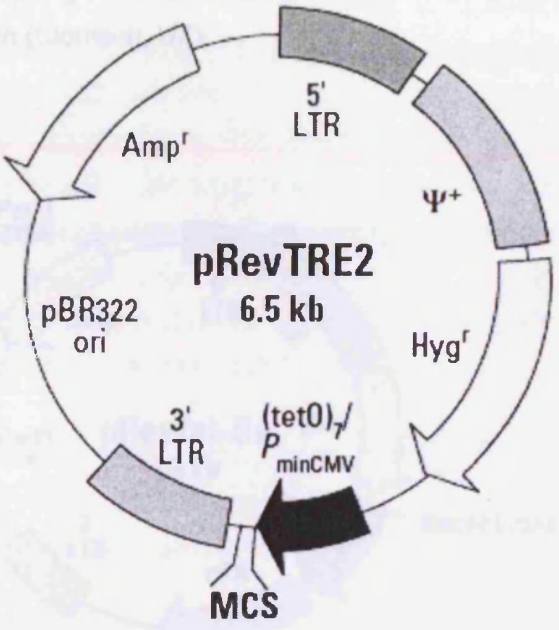


Figure 2.1 Schematic diagram of pBABEpuro-basic vector

Diagram represents circular map of pBABEpuro-basic vector and unique restriction sites within the MCS. Vector components (clockwise from 1): 5' LTR: 5' long terminal repeats; MCS: multiple cloning sites; SV40 IEP: simian virus 40 immediate early promoter; 3' LTR: 3' long terminal repeats; Amp^r: ampicillin resistance gene. *: restriction sites used in the current study. Taken from the Stewartlab website at http://www.stewartlab.net.

2.7.2 pRevTRE2 and pRevTet-on vectors

The pRevTRE2 vectors encoding enhanced green fluorescent protein (pRevTRE2-EGFP) or encoding the 11 amino acids of the C-terminus of Gα subunits (pRevTRE2-$G\alpha_q$, pRevTRE2-$G\alpha_i$, pRevTRE2-$G\alpha_{12}$ and pRevTRE2-$G\alpha_{13}$) were kind gifts from Dr Annette Gilchrist (Caden Biosciences, USA). These minigenes were inserted into the pRevTRE2 vector between its BamHI and HindIII sites (See figure 2.2). The amino acid sequences transcribed by inhibitory mutants were shown in table 2.4.



3329 3339 3349 3359 3369 3379 3389 3399

ACCCGGGGATCCGTCGACGGCCATTATGGCCGTTTAAACTCGAGGCCGCCTCGGCCAAGCTTGCGGCCGCAAACATCGATAAAA

BamH I *Sal I* *Sfi I* *Pme I* *Xho I* *Sfi I* *Hind III* *Not I* *Cla I*

Restriction Map and Multiple Cloning Site (MCS) of pRevTRE2. Unique restriction sites are in bold.

Figure 2.2 Schematic map of pRevTRE2 vector.

Diagram represents circular map of pRevTRE2 vector and unique restriction sites within the MCS. Vector components (clockwise from 1): 5' LTR: 5' long terminal repeats; ψ$^+$: the extended viral packaging signal; Hyg^r: hygromycin resistance gene; $(tetO)_7/P_{minCMV}$: seven direct repeats of the tetO (operator) sequence upstream of a minimal promoter of cytomegalovirus; MCS: multiple cloning site; 3' LTR: 3' long terminal repeats; Amp^r: ampicillin resistance gene.

Vector	Amino acid sequence	Description
pRevTRE2-Gα_q	LQLNLKEYNAV	Gα_q inhibitory mutant
pRevTRE2-Gα_i	IKNNLKDCGLF	Gα_i inhibitory mutant
pRevTRE2-Gα_{12}	LQENLKDIMLQ	Gα_{12} inhibitory mutant
pRevTRE2-Gα_{13}	LHDNLKQLMLQ	Gα_{13} inhibitory mutant

Table 2.4. G protein minigene vectors

pRevTet-on vector encoding the TRE required to drive transcription of pRevTRE2 was obtained from Clontech (Clontech, UK).

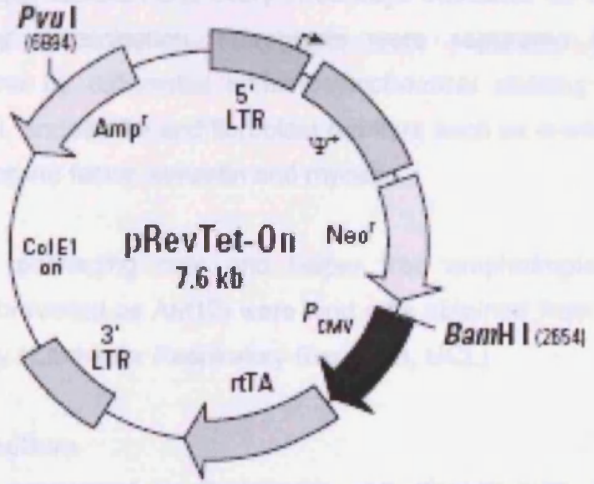


Figure 2.3. Schematic map of pRevTet-On vector

Diagram represents circular map of pRevTet-On and its unique sites. Vector components (clockwise from 1): 5' LTR: 5' long terminal repeats; ψ^+: the extended viral packaging signal; Neo^r: neomycin resistance gene; P_{CMV}: promoter of cytomegalovirus; rtTA: reverse tetracycline-controlled transactivator; 3' LTR: 3' long terminal repeats; Amp^r: ampicillin resistance gene. Image taken from Clontech websit at http://www.clontech.com

2.8 Fibroblasts and retroviral packaging cells

Wild type mouse lung fibroblasts (MLFs) cell line and the PAR_1 deficient (PAR_1 -/-) mouse lung fibroblasts cell line were kind gifts of Dr. Shaun Coughlin (Cardiovascular Research Institute, University of California, San Francisco, CA, USA). These fibroblasts were immortalised through SV40 transformation and used between passage 12 and 25 without any noticeable alteration in phenotype.

Normal primary human adult lung fibroblasts (pHALFs) were a kind gift of Dr. Robin McAnulty from our laboratory and used at passages below 10. These fibroblasts have previously been shown to be high responsiveness to stimulation by coagulation proteinases (Chambers *et al.,* 2000). Primary fibroblasts were initially grown from $1mm^3$ explants dissected from normal human lung tissue. The explants were cultured in DMEM supplemented with 10% FCS. The culture medium was replaced with fresh medium one day after isolation and every three days thereafter for three weeks. Cells were collected by trypsinisation. Fibroblasts were separated by morphological characterisation and by differential immunocytochemical staining for a section of smooth muscle cell, endothelial and fibroblast markers such as α-smooth muscle actin (α-SMA), von willebrand factor, vimentin and myosin.

Phoenix ecotropic packaging cells and helper free amphotropic packaging cells GP^+env AM12 (Abbreviated as AM12) were kind gifts obtained from Dr Sam M Janes from host laboratory (Centre for Respiratory Research, UCL).

2.9 Bacterial cell culture

All vectors were propagated in *Escherichia coli (E.coli)* cells, DH5α-Subcloning Efficiency strain which were obtained from Life Technologies (Life Technologies Ltd, Paisley, UK) and were grown in LB broth or on LB agar plates. Clones were stored at -80°C.

Methods

2.10 Fibroblast cell culture

Mouse lung fibroblast (MLFs), PAR_1 deficient mouse lung fibroblasts and primary human adult lung fibroblasts (pHALFs) were maintained in static culture on 75 cm^2 triangular culture flasks in Dulbecco's modification of Eagle's medium (DMEM), supplemented with penicillin (200 units/ml), streptomycin (200 units/ml), glutamine (4 mM) and 10% (V/V) FCS. Cells were propagated in monolayer cultures in 10%-FCS DMEM in humidified atmosphere of air containing 10% CO_2 at 37 °C. Cells were

passaged by routine trypsinisation protocols when monolayer cultures reached 80-90% confluence. Cells were routinely tested for mycoplasma contamination using MycoAlert Assay (Cambrex, UK) and were negative for mycoplasma infection. If any mycoplasma contamination was detected, all of those cell stocks were disposed of.

Upon reaching visual confluence (4 to 5 days), cells were sub-cultured (passaged) into new culture flasks. The medium was removed and the cell layer was washed twice with 10 ml Ca^{2+} and Mg^{2+} free phosphate buffered saline (PBS) to remove any remaining culture media. Cells were brought into suspension by adding 1 ml of trypsin (0.05%, w/v) / EDTA (0.02%, w/v) solution and incubated at 37°C until cell detachment from the tissue culture plasticware occurred (approximately 3 minutes for fibroblasts). The cells were observed under an Olympus TCK-2 inverted phase contrast light microscope (Olympus Opitcal Ltd, London, UK) to ensure that they had rounded up. Trypsin was neutralised by addition of 9 ml of DMEM supplemented with 10% FCS. The cell suspensions were split at a ratio of 1 to 6. Under these conditions, cells remained viable for about 12 to 25 passages after supply for MLFs, and up to 3-10 passages for pHALFs.

To plate cells for experiments, cells from one tissue culture flask were put in suspension in DMEM-10% FCS and aspirated into a 50 ml sterile polypropylene centrifuge tube (BD Bioscience, UK). They were centrifuged (1000g, 5 min, 4°C) using a bench centrifuge (Sorvall T600B, UK), prior to plating. The supernatant was discarded and the cell pellet was brought into a single cell suspension with 1 ml DMEM-10% FCS by gentle mixing. A further 9 ml culture media was added and an aliquot of the suspension was removed for counting with a sterile pipette. Cells were counted using an improved Neubauer haemocytometer (BDH-Merck, Ltd, Lutterworth, UK) and the density of the cell suspension was adjusted with DMEM-10% FCS.

Cell densities vary from cell to cell. The pHALFs were mainly used for ELISA and were usually seeded in 96-well plates with a density of 8 X 10^3/well. MLFs were applied in multiple experiments with varied densities in different culture plates. The number of MLFs seeded is indicated in Table 2.5.

Tissue Culture Plate	Number of Cells	Volume of Medium	Application
96 well plate	9 x 10^3/ well	100 µl	ELISA
24 well plate	4 x 10^4/ well	500µl	Infection, ELISA
12 well plate	10 x 10^4/ well	1 ml	ELISA, Western Blotting
6 well plate	20 x 10^4/ well	2 ml	Infection, Western Blotting, qRT-PCR

Table 2.5 Mouse lung fibroblast seeding densities for various experimental procedures

2.11 Plasmid manipulations

2.11.1 Transformation of bacteria

For the production of large amounts of cloned DNA, plasmids were transformed into *Escherichia Coli* strain DH5α. For transformation, 10 ng of plasmid DNA was added to 100 µl of competent cell suspension and incubated on ice for 30 minutes. Cells were then subjected to heat shock at 42 °C for 20 seconds before returning to ice for 2 minutes. 900 µl of LB medium was added to cells and incubated at 37 °C for 1 hour, with shaking. 100 µl of suspension was plated onto LB-agar plates containing ampicillin (100 µg/ml) and grown at 37 °C for 16-18 hours.

2.11.2 Plasmid amplification and purification

Protocols for plasmid purification were based on the alkaline lysis method (Birnboim, 1983). This method depends on the fact that high molecular weight linear chromosomal DNA will be denatured when cells are lysed at pH 12.0-12.6, whereas low molecular weight supercoiled plasmid DNA is unaffected. Neutralisation of pH in the presence of high salt concentrations subsequently precipitates chromosomal DNA, which can then be separated from the mixture.

2.11.2.1 Miniprep of Plasmid DNA

Buffers used: Cell Resuspension Solution: 50 mM Tris-HCl, pH 7.5; 10 mM EDTA; 100 µg/ml RNase A; Cell Lysis Solution: 0.2 M NaOH; 1% SDS; Neutralisation Solution: 1.32 M potassium acetate, pH 4.8; Column Wash Solution: 80 mM potassium acetate; 8.3 mM Tris-HCl; 40 µM EDTA.

Minipreparation of plasmids was used to isolate newly constructed plasmids from culture to enable subsequent screening by automated sequencing. Single colonies obtained from transformed bacteria were used to inoculate a 5 ml overnight culture of LB medium containing appropriate antibiotic in a 50 ml loose-capped tube. Solutions and columns supplied with the Wizard® Plus Minipreps DNA Purification System (Promega) were then used. 1.5 ml of culture was poured into a microfuge tube and centrifuged at 10, 000 g for 1 minute. Medium was removed and the dried cell pellet was resuspended in 200 µl of Cell Resuspension Solution by vigorous vortexing. 200 µl of Cell Lysis Solution was added, and mixed gently by inverting tubes 4 times. To precipitate genomic DNA and cell debris, 200 µl of Neutralisation Solution was added, mixed by inverting as above, and centrifuged at maximum speed for 5 minutes. The resulting supernatant was applied to a silica gel Wizard® minicolumn and washed with 2 ml of Column Wash Solution at 10, 000 g for 2 minutes. The minicolumn was transferred to a new microfuge tube and 50 µl of nuclease-free water was added to the column. After 1 minute, the minicolumn was centrifuged again at 10, 000g for 1 minute to elute the plasmid DNA.

2.11.2.2 Maxiprep of Plasmid DNA

Buffers used: Resuspension Solution: 50 mM Tris-HCl, pH 7.5; 10 mM EDTA; 100 µg/ml RNase A; Lysis Buffer: 0.2 M NaOH; 1% SDS; Neutralisation Buffer: 3 M potassium acetate, pH 5.5; Equilibration Buffer: 50 mM MOPS, pH 7; 750 mM NaCl; 15% ethanol; Wash Buffer: 50 mM MOPS, pH 7; 1 M NaCl; 15% ethanol; Elution Buffer: 50 mM Tris-Cl, pH8.5; 1.25 M NaCl; 15% ethanol; TE: 10 mM Tris, pH 8, 1 mM EDTA.

Large quantities of purified plasmids were obtained using the Qiagen® Plasmid Maxi Kit. A single colony was picked from a freshly streaked selection plate and used to inoculate a 5 ml starter culture of selective LB medium, and incubated for ~8 hours at 37 °C on an orbital shaker. The starter culture was then diluted 1:500 into 100 ml of selective LB medium and grown for 12-16 hours at 37 °C on an orbital shaker. Cells were harvested by centrifugation at 6000 g for 15 minutes at 4 °C. Supernatant was discarded and pellets were resuspended in 10 ml of chilled Resuspension Buffer. 10 ml of Lysis Buffer was added to the suspension and mixed gently by inverting. Cells were lysed for 5 minutes at room temperature before stopping the reaction by addition of 10 ml Nuetralisation Buffer and mixed again by inverting. Mixtures were incubated on ice for 20 minutes to allow precipitation of cell debris, genomic DNA and SDS. The sample was then centrifuged at 13,000 g for 1 hour at 4 °C. Subsequently, the supernatant was

passed through a glass wool column to remove any residual precipitate and applied to a Qiagen column equilibrated with 10 ml of Equilibration Buffer and washed twice with 30 ml Wash Buffer. The bound plasmid was eluted in 15 ml Elution Buffer and precipitated by adding 10.5 ml of room temperature isopropanol. Mixtures were centrifugation at 13,000 g for 30 minutes at 4°C and pellets resuspended in 400 µl TE and transferred to a microfuge tube. Plasmid DNA was precipitated by addition of 40 µl of 3 M sodium acetate and 900 µl of ethanol, before mixing and centrifugation at 10, 000 g for 5 minutes. Supernatant was removed and pellets were subsequently washed with 70% ethanol before centrifugation, as above, and resuspension of DNA in 200 µl TE buffer.

2.11.3 Restriction Enzyme Digestion of DNA

Screening of DNA preparations were conducted by restriction enzyme digestions. Master mixes were prepared using appropriate restriction enzymes and their buffers from New England Biolab (UK), and mixed with the plasmid DNA. A typical reaction was set up as detailed in Table 2.6.

Component	Volume
10x 4-CORE or Multi-CORE buffer	2 µl
Enzyme 1	1 µl (10 units)
Enzyme 2	1 µl (10 units)
Plasmid DNA	1-3 µg
Nuclease free water	Make up to 20 µl

Table 2.6 Restriction enzyme digestion master mix recipe.

Digests were incubated at optimal temperature for 2 hours before separation on agarose gels.

2.11.4 Agarose gel electrophoresis of DNA

Following restriction enzymatic digestion, the DNA was separated by agarose gel electrophoresis. During use of agarose gels in this study, it was only necessary to use standard molecular biology grade agarose for DNA separation at different percentages, according to fragment size (see Table 2.7).

Agarose (%)	DNA fragment size range
0.5	700 bp to 25 kb
0.8	500 bp to 15 kb
1.0	250 bp to 12 kb
1.2	150 bp to 6 kb
1.5	80 bp to 4 kb

Table 2.7 Agarose gel percentages used in separation of DNA fragments.

Gels were cast by melting agarose in 1x TBE buffer (90 mM Tris-borate, 2 mM EDTA) containing 0.5 µg/ml of ethidium bromide and pouring into appropriately sized casting trays. Samples were mixed with 6 x loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol, 30% glycerol), loaded into wells and run at 5-10V/cm. Fragments were visualised under UV light and photographed.

2.12 Retrovirus expression system

2.12.1 Retroviral producer cell lines

Phoenix is a second-generation retrovirus producer lines derived from the human embryonic kidney 293 cell line for the generation of helper free ecotropic and amphotropic retroviruses. The cell lines had been created by placing into 293T cells constructs capable of producing gag-pol and envelope protein from ecotropic and amphotropic viruses. For both the gag-pol and envelope constructs non-Moloney promoters were used to minimize recombination potential. Different promoters for gag-pol and envelope were used to reduce their inter-recombination potential. Gag-pol was introduced with hygromycin as the co-selectable marker and the envelope proteins were introduced with diphtheria resistance as the co-selectable marker. AM12 cells were designed in conjunction with pBabePuro vector to reduce the risk of generation of wild type Mo MuL Viruses via homologous recombination (Markowitz *et al.*, 1988).

2.12.2 Cell culture

The Phoenix packaging cells and AM12 cells were propagated in monolayer cultures in DMEM-10% FCS in humidified atmosphere (95% air/ 5% CO_2). For cells transduced with MEK1-pBabePuro constructs, the culture medium was supplemented with 2.5 µg/ml puromycin (100 mg puromycin powder dissolved in 100 ml PBS, sterilized using a 0.22 µm filter and stored at -20 °C before use); for cells transduced with pRevTet-on

vector, the culture medium was supplemented with geneticin (400 µg/ml); for cells transduced with pRevTRE2-EGFP, pRevTRE2-$G\alpha_q$, pRevTRE2-$G\alpha_i$, pRevTRE2-$G\alpha_{12}$ or pRevTRE2-$G\alpha_{13}$, the culture medium was supplemented with 500 µg/ml hygromycin. For cells transduced with both pRevTet-on vector and pRevTRE2-EGFP or G protein minigene vectors, the culture medium was supplemented with both geneticin (400 µg/ml) and hygromycin (500 µg/ml). Culture mediums were regularly replaced with fresh medium every 3 days.

2.12.3 Transduction with MEK1-pBabePuro constructs

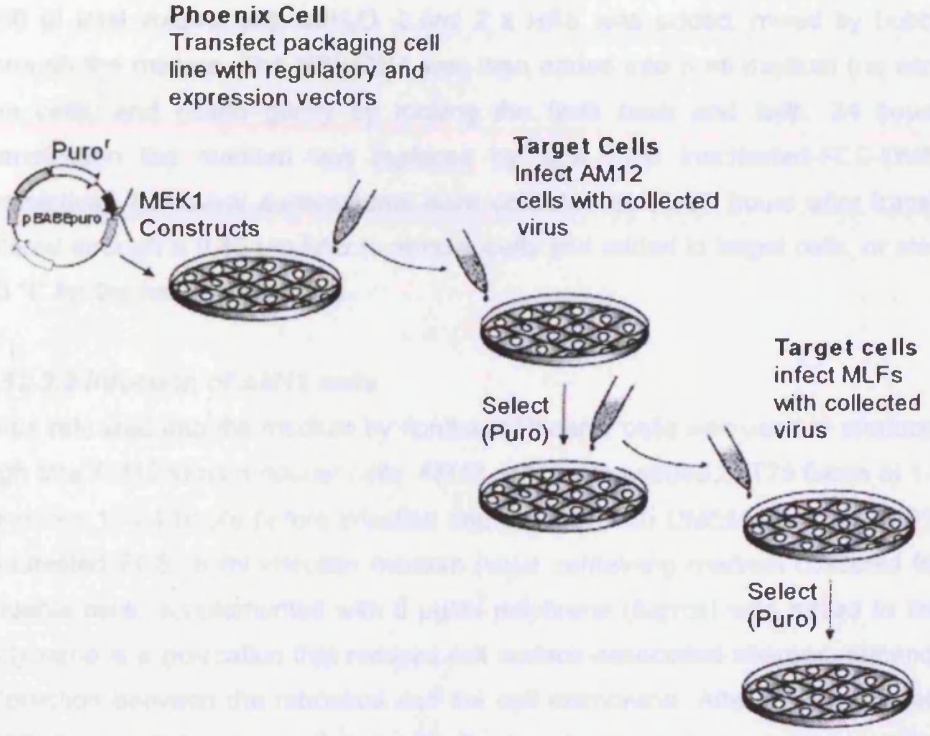


Figure 2.4 Establishing a inducible cell line with MEK1 constructs. The MEK1 constructs were transiently transfected into Phoenix packaging cell line. The resulting virus-containing supernatants are used to infect AM12 cells. High-titer virus producing line was established with puromycin selection. The resulting virus containing supernatants from AM12 were used to infect MLFs and successfully infected MLFs are selected by puromycin.

2.12.3.1 Transient transfection of phoenix Cells

Buffer used: 2 X HBS: 280 mM NaCl, 10mM KCl, 1.5 mM Na_2HPO_4, 12 mM D+Glucose, 50 mM HEPES, pH 7.0.

Ecotropic virus producer cell lines were generated by transfecting the phoenix packaging cells with retroviral vectors. The transfection process used was a calcium phosphate precipitation infection protocol. 80-90% confluent phoenix cells from one T75 tissue culture flask were passaged into four T75 tissue culture flasks with 10% DMEM-FCS without any antibiotics 12-20 hours before the transfection, and the cells shall be 80-90% confluent before transfection. 5 minutes prior to transfection, cells in the flask were washed twice with DMEM and supplemented with 5 ml DMEM only; chloroquine (25 µM) was next added to each flask. Chloroquine increases the transfection efficiency by neutralizing vesicle pH and thereby inhibiting lysosomal DNAse. 10 µg DNA, 438 µl ddH_2O, and 61 µl 2 M $CaCl_2$ were mixed and brought to a 500 µl total volume with ddH_2O. 0.5ml 2 x HBS was added, mixed by bubbling air through the mixture. The HBS/DNA was then added into 5 ml medium (no serum) on the cells, and mixed gently by rocking the flask back and forth. 24 hours post-transfection the medium was replaced by 10% heat inactivated-FCS-DMEM (no antibiotics). Retroviral supernatants were collected at 65-90 hours after transfection, filtered through a 0.45 µm filter to remove cells and added to target cells, or stored at -80 °C for the next experiments.

2.12.3.2 Infection of AM12 cells

Virus released into the medium by confluent Phoenix cells was used to produce stable high titre AM12 virus producer cells. AM12 cells were seeded in T25 flasks at 1-2 x 10^5 densities 12-24 hours before infection and cultured with DMEM containing 10% heat-inactivated FCS. 5 ml infection medium (virus containing medium collected from the phoenix cells; supplemented with 5 µg/ml polybrene (Sigma) was added to the cells. Polybrene is a polycation that reduces cell surface-associated charges, enhancing the interaction between the retrovirus and the cell membrane. After 12 hours infection at 37°C the medium was replaced with fresh culture medium containing 10% heat-inactivated FCS). The selection medium containing puromycin (2.5 µg/ml) was used 48 hours later and changed every 3 days until cells reached confluence. After 1 week selection, cells were passaged into T75 flasks. When cells reached 80% confluence, the selection medium was replaced with 5 ml DMEM containing 10% heat-inactivated FCS in order to collect high titer viral supernatants. Retroviral supernatants were collected 24 hours later, filtered through a 0.45 µm filter to remove cells and added to target cells, or stored at -80 °C for the next experiments.

2.12.3.3 Infection of mouse lung fibroblasts

MLFs cells were seeded onto 24-well plate 12-20 hours before infection. 500 µl supernatant containing the virus from confluent AM12 cells was mixed with polybrene (5 µg/ml) and placed on MLFs, following incubation for 24 hours at 37°C. The media were then replaced with 10% FCS-DMEM for another 24 hours. Cells were then selected with the selection medium containing puromycin (2.5 µg/ml). Relative experiments were carried out when the selected cells reached confluence.

2.12.4 Transduction with pRevTRE2 vectors

For inhibition of G-protein signalling by minigenes, the RevTet-On expression system (BD Biosciences) was employed (Figure 2.5). The RevTet-On system uses the pRevTet-On and pRevTRE response vector. The pRevTet-on vector encodes the reverse tetracycline sensitive regulatory element (rtTA) consisting of a reverse Tet repressor (rTetR) and VP16 activation domain. In the presence of doxycycline, this protein interacts with the pRevTRE response vector, binding at the Tet response element (TRE), initiating transcription of the gene of interest. In the current study, G-protein minigenes were cloned into the upstream multiple cloning site (MCS). The TRE contains seven direct repeats of the 42bp tetO sequence and the minimal immediate early promoter of cytomegalovirus (P_{minCMV}).

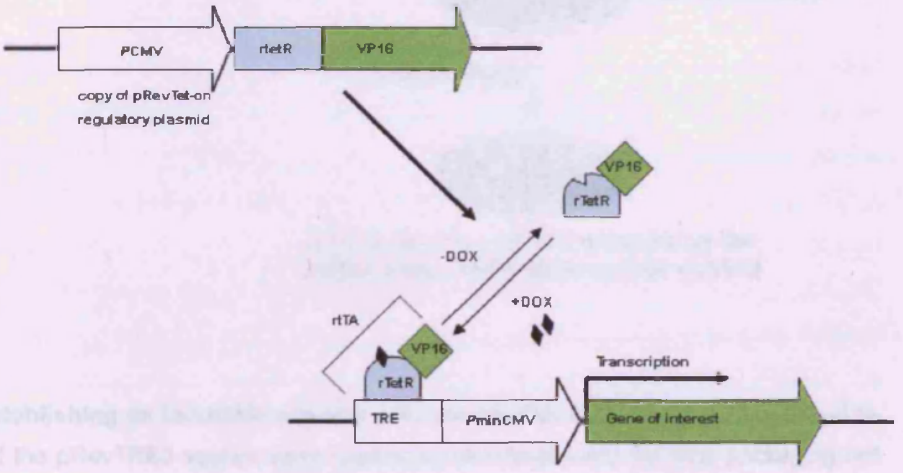


Figure 2.5 Mechanism of BD RevTet-On™ gene expression. In the presence of doxycycline, the rtTA binds to the TRE which drives the transcription in the presence of Dox. (Taken from BD RevTet™ System User Manual, BD Biosciences).

2.12.4.1 Constructing a stable expression cell line.

To create a stable cell line that expresses the gene of interest upon induction, a series of transfection and infection as diagrammed in Figure 2.6.

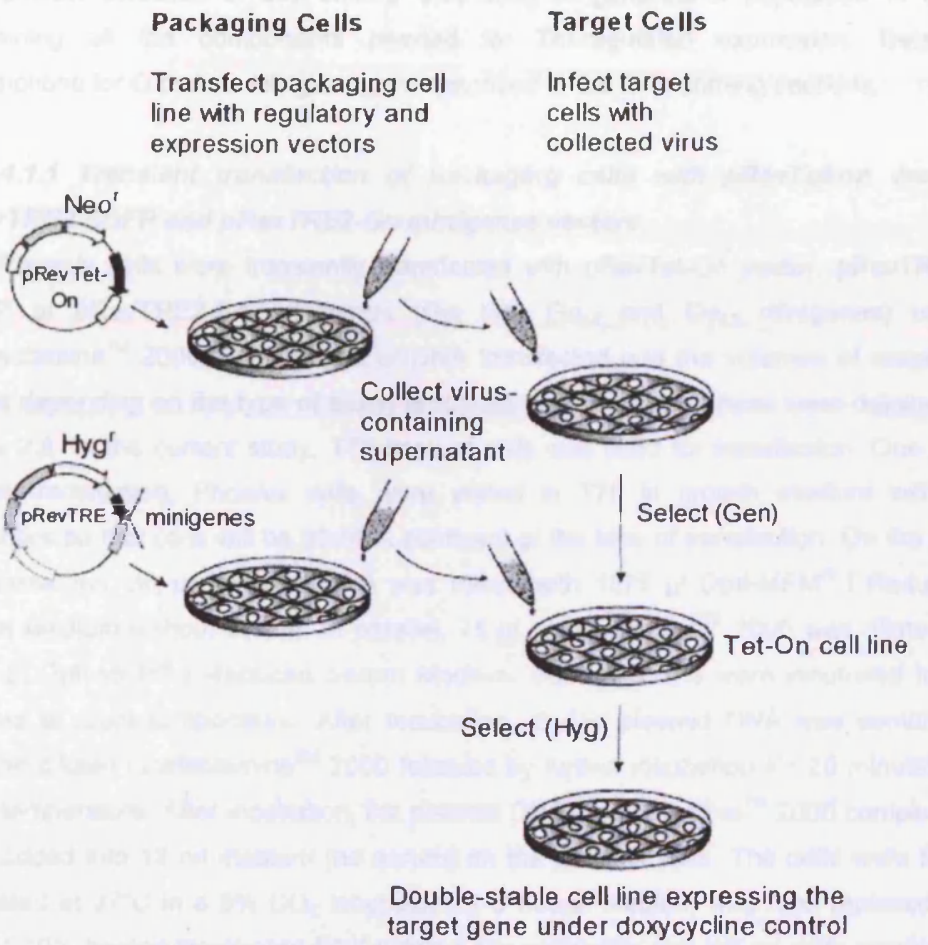


Figure 2.6 Establishing an inducible cell line with the BD RevTet™ System. The pRevTet-On vector and the pRevTRE2 vectors were separately transfected into the viral packaging cell line. The resulting virus-containing supernatants were used to serially infect target cells: first with pRevTet-on virus to produce a Tet-On cell line, then with pRevTRE virus to integrate the G protein minigenes. Expression is induced by the addition of doxycycline. (Modified from BD RevTet™ System User Manual, BD Biosciences)

Briefly, the first step was to make two different retroviruses: one to deliver the pRevTet-On regulator construct to the target cells and the other to deliver G protein minigenes.

To produce the virus, pRevTet-On vector and pRevTRE2-minigenes were transfected into the phoenix cells in parallel. When packaging is complete, supernatant containing pRevTet-On virus was used to infect the target cells first. Geneticin selection of this culture was next applied in order to obtain a population of cells that have integrated rtTA. Next the Tet-On cell line was infected with pRevTRE2-minigene virus. Hygromycin selection of this culture was used to generate a population of cells containing all the components needed for Tet-regulated expression. Detailed descriptions for G protein minigenes are described in the forth coming sections.

2.12.4.1.1 Transient transfection of packaging cells with pRevTet-on vector, pRevTRE2-EGFP and pRevTRE2-Gα minigenes vectors

The Phoenix cells were transiently transfected with pRevTet-On vector, pRevTRE2-EGFP or pRevTRE2-Gα minigenes ($G\alpha_i$ $G\alpha_q$ $G\alpha_{12}$ and $G\alpha_{13}$ minigenes) using Lipofectamine™ 2000. The amount of DNA transfected and the volumes of reagents varied depending on the type of assay and plate type used, and these were detailed in **Table 2.8**. In the current study, T75 flask of cells was used for transfection. One day before transfection, Phoenix cells were plated in T75 in growth medium without antibiotics so that cells will be 80-90% confluent at the time of transfection. On the day of transfection, 30 µg plasmid DNA was mixed with 1875 µl Opti-MEM® I Reduced Serum Medium without serum. In parallel, 75 µl Lipofectamine™ 2000 was diluted in 1875 µl Opti-MEM® I Reduced Serum Medium. Both solutions were incubated for 5 minutes at room temperature. After incubation, diluted plasmid DNA was combined with the diluted Lipofectamine™ 2000 followed by further incubation for 20 minutes at room temperature. After incubation, the plasmid DNA-Lipofectamine™ 2000 complexes were added into 12 ml medium (no serum) on the phoenix cells. The cells were then incubated at 37°C in a 5% CO_2 incubator for 6 hours. Medium was next replaced by DMEM-10% heated-inactivated FCS without any antibiotics and left on cells overnight. Virus containing supernatants were collected at 65-90 hours after transfection, filtered through a 0.45 µm filter to remove cell debris and added to target cells, or stored at -80 °C for the next experiments.

Culture vessels	Surface of area per well	Volume of plating medium	Volume of dilution medium	DNA	Lipofectamine™ 2000
96-well	0.3 cm^2	100 µl	2 ×25 µl	0.2 µg	0.5 µl
24-well	2 cm^2	500 µl	2 ×50 µl	0.8 µg	2 µl
12-well	4 cm^2	1 ml	2 ×100 µl	1.6 µg	4 µl
6-well	10 cm^2	2 ml	2 ×250 µl	4 µg	10 µl
60-mm	20 cm^2	5 ml	2 ×500 µl	8 µg	20 µl
10-cm	60 cm^2	15 ml	2 ×1.5 ml	24 µg	60 µl
T75 flask	75 cm^2	18 ml	2 ×1.875 ml	30 µg	75 µl

Table 2.8 Volumes of reagents used in Lipfectamine™ 2000 transfection

2.12.4.1.2 Producing a stable Tet-On-MLF cell line

MLFs were seeded in T75 12-18 hours before infection so that MLFs will be 30-50% confluent at the time of infection. After 24 hours incubation, virus-containing supernatant from phoenix cells were then collected and applied on MLFs seeded 12-18 hours before the infection. 24 hours after infection, the MLFs were rescued by replacing the virus-containing medium with DMEM containing 10% heat-inactivated FCS without any antibiotics. After 24 hours further incubation, the MLFs were then selected with 400 µg/ml geneticin. The selection medium containing 400 µg/ml geneticin was changed every 3 days until cells reached confluence. Minimum of one week selection was carried out before used for subsequent experiments or frozen down as stock.

2.12.4.1.3 Infection of Tet-On-MLF cell line with pRevTRE2-EGFP and pRevTRE2-Gα minigenes

The Phoenix cells were transiently transfected with pRevTRE2-EGFP and pRevTRE2-Gα minigenes ($G\alpha_i$, $G\alpha_q$, $G\alpha_{12}$ and $G\alpha_{13}$ minigenes) in parallel as described in ***Section 2.12.4.1***. The virus-containing supernatants were collected and applied onto the Tet-On-MLFs cells seeded 12-18 hours before the infection. 24 hours after infection, the cells were rescued by replacing the virus-containing medium with DMEM containing 10% heat-inactivated FCS without any antibiotics. After the rescue, cells were selected with both 400 µg/ml geneticin and 500 µg/ml hygromycin for a week. Relative experiments were carried out when the cells survived from selection and reached confluence, with the addition of 3 µg/ml of doxycycline in culture medium in order to induce the expression of gene of interest.

2.13 Enzyme-linked immunoassay (ELISA)

2.13.1 Cell conditions and cell supernatants collection

Cells were seeded 48 hours before assay. In order to avoid edge effects, cells were only dispensed into the central 10 × 6 wells of the 96-well plate. The outer wells were filled with 100 µl DMEM-5% FCS only. After 24 hours in culture and before confluence, cells were washed with serum-free DMEM twice, and the cells were quiesced in 0.1% FCS-DMEM (MLFs) or serum-free DMEM (pHALFs) and used at sub-confluence. Before each experiment, the starvation medium was removed and replaced with fresh serum-free DMEM, with or without mediators or inhibitors for a selected length of time (details please refer to individual experiment described in results section). Serum-free conditioned media were then collected from cell culture plates, spun to remove cell debris. Samples were used for ELISA immediately, or stored in -80°C and defrosted on ice before ELISA.

2.13.2 Murine CCL2 ELISA

Buffers used: Coating buffer: 0.2 M sodium phosphate, PH 6.5; Assay dilute: PBS with 10% heat-inactivated FBS; Washing buffer: PBS with 0.05% Tween-20; Stop solution: 1 M H_3PO_4.

ELISA: Microtest™ 96-well ELISA plates were obtained from BD Biosciences, Pharmingen (San Diego, CA, USA). The assay was reported to recognise total murine CCL2. The lowest reference on the standard curve 15.6 pg/ml and the highest was 1000 pg/ml. The protocol followed was identical to that in the accompanying literature. Briefly, the Microtest™ 96-well ELISA plates were coated with 50 µl of anti-mouse CCL2 capture antibody (diluted 1:250 in coating buffer) and were then left overnight at 4°C. After overnight incubation, plates were washed and were next blocked with 100 µl assay dilute for 1 hour at room temperature. After the incubation, plates were again washed, 50 µl of each sample and serial dilutions (0-1000 pg/ml) of standard CCL2 protein were added to each well accordingly and incubated for 2 hours at room temperature. Following incubation, plates were then washed, 50 µl of the anti-mouse CCL2 detecting antibody (diluted 1:250 in assay diluents) was added to each well for 1 hour at room temperature. After further washes, plates were finally incubated with 50 µl per well of the substrate solution for 30 minutes at room temperature. 25 µl of stop solution was subsequently added to each well after 30 minutes incubation. Plates were next read at A1 450nm, A2 550nm with a plate reader (Multiskan MCC/340, Titertek).

2.13.3 Human CCL2 ELISA

Buffers used: Bicarbonate buffer (pH9.6): 1.59g Na_2CO_3, 2.93g $NaHCO_3$ and 0.2g NaN_3 in 1L double-distilled water, filtered and stored at room temperature; Blocking buffer: 1% BSA in PBS; Coat and Block wash buffer (C/B wash): 0.1M KPO_4 and 0.05% Tween20; Assay wash buffer: PBS with 0.05% Tween-20; Stop solution: 1 M H_3PO_4.

ELISA: The assay was reported to recognise total human CCL2. The lowest reference on the standard curve 34 pg/ml and the highest was 2150 pg/ml. Briefly, dissolve anti-human CCL2 capture antibody in bicarbonate buffer (1:250). Apply 50 µl aliquots to each well and incubate at room temperature for a minimum of 5 hours. Wash the plate with C/B wash buffer and add 100 µl blocking buffer to each well. Incubate the plate with blocking buffer overnight at 4 °C. Make up standards or dilute samples with 0.1% BSA in PBS. Apply 50 µl aliquots of each standards and samples to each well and incubate at room temperature for 2 hours. Plates were then washed with assay wash buffer. Dilute anti-human CCL2 detecting antibody in blocking buffer (1:250) and add 50 µl aliquots to each well followed by 2 hours incubation at room temperature. Plates were next washed with assay wash buffer. Dilute streptavidin-HRP 1:2000 in 0.1% BSA in PBS and add 50 µl aliquots to each well. Incubate plates at room temperature for 1 hour followed by further wash with assay wash buffer. Plates were finally incubated with 50 µl per well of the substrate solution for 30 minutes at room temperature. Final reaction was terminated by adding 50 µl of stop solution in each well and kept in dark at room temperature for 30 minutes. Plates were next read at A1 450nm, A2 550nm with a plate reader.

2.14 Western blotting

Western Blotting was used in order to study the expression levels of proteins of interest. Samples to be analysed were solubilised with detergents and reducing agents and separation of proteins from crude lysate was achieved by SDS-PAGE. Transfer onto a PVDF membrane followed by chemiluminescnet detection was carried out for specific protein analysis, such as detection and quantitation. Using specific antibodies, amounts and levels of protein phosphorylation were determined.

2.14.1 Preparation of Buffers:

Washing buffer: PBS with 0.2 M Na_3VO_4; 0.5 M NaF; 1 x Lysis buffer: 50 mM Tris (pH 8), 1% Triton X 100, 100 mM NaCl, 1 mM $MgCl_2$, 1 mM Na_3VO_4, 20 mM NaF, 1 mM β-glycerophosphate, 1 tablet of protease inhibitor cocktail in 10 ml lysis buffer. Laemmli buffer: 100mM dithiothreitol (DTT), 0.005% bromophenol blue; 1 x running buffer: 0.025

M Triz, 0.19 M Glycine, 0.1% SDS; Transfer buffer: 25 mM Tris, 0.2 M glycine, 1% SDS, 20% methanol; 1 x TBS-Tween: 10 mM Tris (pH 8), 150 mM NaCl, 0.1% Tween 20; Blocking buffer: 5% w/v non-fat milk powder in 1 x TBS-Tween.

2.14.2 Preparation of cell extracts

MLF cells were seeded 24 to 48 hours prior to subsequent treatment. After each treatment (each result section will contain the particular experimental details), cells were lysed. Specifically, cells were washed twice with ice-cold washing buffer. Cells from 6-well plates were kept on ice and lysed in 200 µl of lysis buffer supplemented with a complete protease inhibitor cocktail ('complete-Mini' pellets from Roche, UK). Viscous lysates were scraped and collected into labelled microfuge tubes, and placed on ice followed by spinning for 10 minutes at 13, 000g in order to remove cell debris. The chromosomal DNA was sheared by repeated passaging through a hypodermic needle (25G) for 25 times per sample. Whole cell extracts were stored in -80 °C before use.

2.14.3 BCA Protein assay

Protein concentrations of samples were assessed according to the read out of BCA Protein Assay (bicinchoninic assay, *pierce, USA*). The BCA assay works by the biuret reaction whereby protein reduces Cu^{2+} to Cu^{1+} in an alkaline medium. Each Cu^{1+} ion then complexes with 2 molecules of bicinchoninic acid to form a water-soluble purple-coloured reaction product that exhibits a strong absorbance at 562 nm which is linear with increasing protein concentration over a broad working range from 20 µg/ml to 2000 µg/ml. The amount of protein in samples was derived by comparison a bovine serum albumin standard curve. The assay was performed by adding 200 µl of freshly prepared BCA working reagent to 20 µl of each sample or standard (assayed in duplicate) in a 96-well plate. The plate was covered with plate seal and agitated on a plate shaker for 30 seconds followed by 30 minutes incubation at 37 °C. The plate was then cooled to room temperature and read at 540 nm on a Titertek Multiscan MCC/340 plate reader (*Labsystems, Finland*).

2.14.4 Separation of proteins by SDS-polyacrylamide gel electrophoresis

SDS-PAGE gels were prepared according to the recipes outlined in Tables 2.9 and 2.10 and cast using the Bio-Rad Mini-Protean II casting apparatus. For electrophoresis, an aliquot of each sample (equivalent to 5 µg of protein for the detection of ERK1/2, 30 µg for detection of p38 and c-Raf, 100 µg for the detection of MLC and $G\alpha_i$) was added with laemmli buffer (5% in the final mix) and incubated for 10 minutes at 80 °C and then

returned to the ice until they were loaded onto a SDS-PAGE gel: 10% SDS-PAGE gel for the detection of ERK1/2, p38 and c-Raf which contained 10% resolving gel and 5% stacking gel; 15% SDS-PAGE gel for the detection of MLC which contained 15% resolving gel and 5% stacking gel, and 12.5% SDS-PAGE gel for the detection of $G\alpha_i$ which contained 12.5% resolving gel and 5% stacking gel. The resolving gel mixture was overlaid with water-saturated butan-2-ol while the TEMED and APS (Ammonium persulphate)-catalysed polymerisation occurred. This was to achieve a smooth and level surface when the gel was set. The butan-2-ol was thoroughly washed off and a stacking gel containing loading wells was poured above the set resolving gel.

	5%	10%	12.5%	15%	20%
1.5 M Tris pH 8.9	2.5ml	2.5ml	2.5ml	2.5ml	2.5ml
30%, 37:1 acryl/bisac	1.7ml	3.3ml	4.2ml	5.0ml	6.7ml
Water	5.7ml	4.1ml	3.2ml	2.4ml	0.7ml
20% SDS	100 µl	100 µl	100 µl	100 µl	100 µl
10% APS	67 µl	67 µl	67 µl	67 µl	67 µl
TEMED	4 µl	4 µl	4 µl	4 µl	4 µl

Table 2.9 SDS-PAGE resolving gel recipes

	10 ml	5 ml
1 M Tris pH 6.8	1.25 ml	0.63 ml
30% Acryl/bis	1.6 ml	0.8 ml
Water	7.0 ml	3.5 ml
20% SDS	100 µl	50 µl
10% APS	67 µl	33 µl
TEMED	10 µl	5 µl

Table 2.10 SDS-PAGE stacking gel recipes.

Samples prepared as above, were loaded onto the SDS-polyacrylamide gels. A pre-stained 4 kDa apparent molecular mass (*Mr*) ladder (SeeBlue TM; Novex Inc./R&D Systems Europe Ltd, Abingdon, UK) was run in a separate lane to easily identify various *Mr*. Samples were electrophoresed at 120V for 2hours in running buffer.

2.14.5 Transfer of proteins onto PVDF membrane

Resolved proteins were transferred to a polyvinylidene difluoride (PVDF) membrane using a horizontal semi-dry transfer method (multiphor II; *Pharmacia LKB, Sweden*). The PVDF membrane was pre-equilibrated in transfer buffer for at least 10 minutes prior to blotting. All other components were also soaked beforehand in transfer buffer. PVDF membrane was placed on top of eight pieces of Whatmann 3MM filter paper and overlaid with the gel. Air bubbles were removed by rolling over the membrane using a pipette. A second layer of filter paper was placed on top of the gel to complete the sandwich. The cassette assembly was placed into a tank and a current of 40 mA/membrane was applied for one and half hours.

2.14.6 Detection of proteins by ECL

Following transfer, the membrane was probed with specific antibodies and proteins detected by chemiluminescence. Specifically, the membrane (bearing the covalently bound proteins) was incubated in blocking buffer for 1 hour at room temperature on a rotating platform. The membrane was then incubated overnight at 4°C in 0.25% BSA buffer containing an appropriate dilution of antibody for the protein of interest. Membranes were washed 3 x, 5-minute each, at room temperature in TBS-tween (10 mM Tris, pH 8, 150 mM NaCl, 0.1% Tween 20) and then incubated for 1 hour at room temperature with a 1:2500 dilution of horseradish peroxidase (HRP)-linked secondary antibody in 0.25% milk buffer (Santa Cruz Biotechnology). Following 3 x, 5-minute washes in 0.5% tween-TBS, blots were developed by enhanced chemiluminescence (ECL) according to manufacturer's instructions (Amersham). Exposure time was adjusted accordingly to ensure accurate quantification of protein bands. Immunoreactive bands were visualized by exposing the membrane to autoradiography film developer (Kodak, UK). A list of all antibodies used in this study for western blotting is detailed in **Table 2. 2.**

2.14.7 Quantification of protein bands

Semi-quantitative analysis of western blots was performed using densitometry. The blots underwent transmissive greyscale scanning at 300 dpi on a standard flatbed scanner (Epson, UK). The scanned images were transferred to the public domain NIH

image 1.61 program (developed at the U.S. National Institute of Health and available on the internet at http://rsb.info.nih.gov/nih-image/ and the optical density of each protein band was calculated with reference to a calibration curve. This calibration curve was specifically generated by scanning a Kodak photographic Step Tablet (Kodak, UK) with known optical density gradient using the same settings as described above. The obtained optical density for the activated protein of the target gene was then normalised against the optical density for the total protein of the same target gene, allowing correction for protein loading and therefore meaningful comparison between samples.

2.15 mRNA study

2.15.1 RNA isolation, purification and quantitation

Cells were seeded in 6 well tissue culture plates in DMEM-10% FCS prior to subsequent treatment. Each result section will contain the particular experimental details. Following stimulation, the media was removed and the cell layer was scraped into 1 ml of TRIzol. TRIzol is a phenol-based reagent for single-step isolation of RNA from cells. It is a solution of phenol and guanidine isothiocyanate which disrupts cell membranes by dissolving cell components but maintaining the integrity of RNA. Total RNA was isolated according to the manufacturer's instructions (*Invitrogen, UK*). The homogenate was incubated at room temperature for 10 minutes, followed by the addition of 200 µl chloroform and centrifugation at 13,000 g for 15 minutes at 4°C. The supernatant was aspirated into a new tube, with 500µl isopropanol added to each sample, centrifuged at 13,000 g for 10 minutes at 4°C. Supernatants were then discarded and the pellets were washed with 1 ml 80% ethanol. Samples were centrifuged at 13,000 g for 10 minutes at 4°C and the supernatants aspirated. Samples were centrifuged at 13,000 g for 1 minute at room temperature again in order to remove as much remaining liquid as possible. Pellets were partially air-dried (about 30 minutes) until a clear gel obtained and re-suspended in 20 µl DEPC-treated water.

The samples (2 µl each) were mixed with H_2O (5 µl for each sample), and RNA loading buffer containing ethidium bromide (3 µl). The mixture was then heated to 65°C for 5 minutes, prior to loading onto an agarose gel to check RNA quality. To prepare a 1% agarose gel, 2 g of agarose were added to 162 ml DEPC-treated water and heated in a microware. After cooling the agarose solution down to about 65°C, formaldehyde to 9% (v/v; about 18 ml) and 20 ml of 20 X MOPS (0.4 M 3-N-morpholino-propane sulphonic acid, 32 nM sodium acetate and 4 mM EDTA), were added. The gel was then cast into a gel tray, containing a loading comb and left to set at room temperature. The gel was

finally submerged in running buffer (1 X MOPS). RNA samples were loaded into the wells of the agarose gel and separated by electrophoresis in an electrophoresis chamber (International Biotechnologies Inc., CT, USA) at a constant voltage of 70 V for 1 hour. RNA loading and integrity were confirmed by the presence of the ethidium bromide stained 18S and 28S ribosomal RNA bands, which were visualised with a Syngene Gene Bio-imaging system (Synoptics, UK).

In order to avoid DNA interference in the final qRT-PCR experiments, RNA purification was carried out for any samples that have been proofed to be in a good condition by electrophoresis described above. Total RNA was treated with DNase to remove contaminating genomic DNA, using DNA-free™ kit (Ambion Ltd. UK). 17 µl of each sample from above was added with 2 µl DNase buffer and 1 µl rDNase following incubation at 37°C for 30 minutes. Further 2 µl DNase inactivation reagent was added and samples were incubated 5 minutes at room temperature, mixed twice during the incubation period. Samples were centrifuged at 10,000 g for 2 minutes at RT, the purified RNA were transferred to a fresh tube and stored at -70°C until used.

The purified RNA was again analysed by running samples on an agarose gel as described above. RNA was next quantified in order to prepare equal amount of RNA of each sample for cDNA synthesis. For RNA quantification, 2 µl of each RNA sample was diluted in 50 µl ddH_2O. The absorbance of each sample was sequentially measured at a wavelength of 260 nM and 280 nM using a UV spectrophotometer (Ultraspec 3000, Pharmacia Biotech, Buckinghamshire, UK). The ratio of the optical density units at 260 nM and 280 nM was used as a gross indicator of the purity of the RNA sample. Ratios between 1.7 and 1.9 were considered acceptable. RNA concentration was calculated based on the assumption that 40 µg of total RNA in 1 ml water gives an absorbance reading of 1 optical density unit at 260 nM. The volume of the RNA sample containing 3 µg total RNA from fibroblast cultures was calculated and made up to a total volume of 10 µl with DEPC-treated water.

2.15.2 cDNA synthesis

cDNA was prepared by reverse transcription (RT) using a RT-PCR kit from Applied Biosystems (Roche, Lewes, UK). Reactions were performed according to the manufacturer's instructions. Briefly, 3 µl RNA (equivalent to 1 µg) from above was added to the reaction mix (4 µl $MgCl_2$; 2 µl 10 x Buffer; 8 µl dNTP mix; 1 µl reverse transcriptase). Reactions were then performed using a tetrad (PTC-225, Peltier Thermal cycle) according to the manufacturer's instruction: incubation at room

temperature for 10 minutes, 42°C for 15 minutes, 99°C for 5 minutes and then 5°C for 5 minutes. Samples were stored at -20°C before use.

2.15.3 RT-PCR

RT-PCR was performed using a tetrad (PTC-225, Peltier Thermal cycler, Global Medical Instrumentation, USA). For each pair of primers, a master mix was prepared which contained all reagents except the cDNA. The final volume of each PCR reaction was 25 µl, containing cDNA, AmpliTaq DNA polymerase, buffer, dNTPs, magnesium chloride (all from *Applied Biosystems, UK*) and 0.25 µM of each primer. The following cycling conditions were used: 94 °C 5 minutes; 94 °C 30 seconds for 35 cycles; 60 °C 30 seconds; 72 °C for 30 seconds; 72 °C for 7 minutes and 25 °C for 30 seconds.

10 µl of each PCR reaction was added to 5 µl of loading buffer (40% w/v sucrose; 0.25% w/v bromophenol blue; 0.25% w/v xylene cyanol, made up in distilled water) and electrophoresed through a 2% agarose gel containing 0.5 µg/ml ethidium bromide. Bands were visualised by UV transilumination. The size of the PCR products was estimated using a co-migrated DNA size marker (Roche Diagonostics).

2.15.4 Quantitative Real time RT-polymerase chain reaction (qRT-PCR)

Quantitative real time RT-PCR was conducted using the Platinum SYBR Green qPCR SuperMix UDG (*Invitrogen, UK*) with 1 ng of cDNA and forward and reverse primers each at a final concentration of 500 nM, on a LightCycler 1.5 Real-time Detection System following the manual and analysed using LightCycler Real-time PCR Detection System Software Version 3.5 (all from Roche, Hertfordshire, UK). Cycling conditions were as follow: one cycle of 50 °C (2 minutes), 95 °C (2 minutes); 50 cycles of 95 °C (5 seconds), 55 °C (5 seconds), 72 °C (15 seconds). Melting curve analysis was used to confirm the specificity of the PCR product.

The magnesium concentration for each primer set was then optimised. A number of real time RT-PCR reactions were performed in parallel, using template cDNA from a sample known to express the gene of interest but substituted with varying concentrations of Mg^{2+}. Reactions with the steepest logarithmic amplification plot, and typically also the lowest cycle number at which detectable amplification occurred, indicated the optimal Mg^{2+} concentration for use with the given primer set.

The efficiency of each primer pair was also assessed by determining crossing point (Cp) values for RT-PCR reactions using a series of half logarithmic dilutions of template

cDNA. Cp values were defined as the earliest point of the linear region of the logarithmic amplification plot reaching a threshold level of detection. Log concentrations of samples were plotted against Cp values, and the slope of the plot determined. Efficiency was then given by the equation: Efficiency $=10^{(-1/slope)}$. Primers were only used if the PCR efficiency was greater than 95%.

To examine the quantitative differences in the mRNA target gene expression in each sample, PCR product was measured at the end of each cycle. Cp values were determined from the linear region of the logarithmic amplification plots. Each sample was also tested for expression of the 'houskeeping' gene 18S and this was used to normalise between samples. Relative quantitation was calculated according to the $E^{-\Delta\Delta CP}$ method. Briefly, ΔCP=Cp target gene-Cp 18S. All the samples were then compared to the control (untreated group). The $\Delta\Delta CP=\Delta CP$ – Average ΔCP control. Primers with efficiency over 1.8 were used. The fold increase$=1.8^{-\Delta\Delta CP}$.

2.16 Immunocytofluorescence staining

Immunocytofluorescece staining was used to recognize the intracellular CCL2 protein production. MLFs were plated on an 8-well chamber glass-slides in a density of 3×10^4 / well. After 24 hours, cells were starved in 0.1% FBS-DMEM for 24 hours. Following stimulation (details please see individual results section), cells were rinsed with PBS gently and then fixed for 15 minutes with 4% formaldehyde in PBS. Following fixation, cells were then rinsed with ice-cold sterile PBS for 3 times, 10 minutes each. After washing, cells were permeabilized with 0.2% Triton X-100 at room temperature for 10 minutes. Cells were then rinsed again with ice-cold sterile PBS for 3 times, 10 minutes each. To avoid non-specific binding, the cells were then incubated with 4% normal rabbit serum for 1 hour at room temperature. After 1 hour incubation with normal rabbit serum, cells were incubated with primary antibody (goat polyclonal anti-CCL2, R&D) for 1 hour at room temperature following three washes with ice-cold sterile PBS, 10 minutes each. Secondary antibody (FITC-conjugated rabbit anti-goat IgG) was incubated in the dark for 1 hour at room temperature and subsequently slides were washed three times with ice-cold sterile PBS, 10 minutes each. Finally, the cells were stained with DAPI for 10 minutes in dark followed by two washes with ice-cold sterile PBS, 5 minutes each. The chambers slide was then mounted with Gevotol and covered with cover slip, left overnight at room temperature in dark. Slides were analyzed the following day.

Immunocytofluoresence images were acquired by confocal microscopy using the Bio-Rad MRC 1024 confocal system (Hercules, CA), and the images were analyzed for the pixel intensity of the fluorescence using the Bio-Rad LaserPix image analysis software.

2.17 Data handling and statistical analysis

All data are presented as mean ± standard errors of the mean (SEM) from three triplicate cultures, unless otherwise indicated. Statistical evaluation was performed using an unpaired t-test for single group comparisons and Newman-Keuls one way analysis of variance (ANOVA) for multiple group comparisons. The mean values of various parameters were considered to be significantly different when the probability of the differences of that magnitude, assuming the null hypothesis to be correct, fell below 5% (i.e. $p<0.05$).

Chapter 3: Results (I)

3.1 Role of PAR_1 activation in mouse lung fibroblast CCL2 protein production in vitro

The aim of the first studies was to determine whether thrombin has the potential to influence the production of CCL2 by wild type mouse lung fibroblasts (MLFs), and to identify the signalling receptor involved.

3.1.1 Effect of thrombin on CCL2 protein production by mouse lung fibroblasts

In order to determine the effect of thrombin on MLF CCL2 production and release, MLFs were exposed to various concentrations of thrombin, and CCL2 protein levels in culture supernatants were assessed by ELISA. Previous studies from Bachli and colleagues showed that thrombin induces a significant increase of CCL2 protein production after 6 hours incubation in dermal fibroblasts (Bachli *et al.*, 2003). Hence, a 6 hour incubation period was used to examine the concentration-dependent effect of thrombin in MLFs. Figures 3.1A & B show that thrombin stimulated CCL2 protein release in a time- and concentration-dependent manner from 0.03 nM onwards. CCL2 production continued to increase at concentrations higher than 0.03 nM and the effect did not plateau at the highest concentration of thrombin (300 nM) examined. Time-course experiments (Figure 3.1B) with thrombin at a physiologically relevant concentration (10 nM) showed that the significant increase was observed from 2 hours onwards, and the sharpest increase in CCL2 release occurs over the first 12 hours. For comparison, the basal CCL2 protein production did not change over the 24 hours incubation period

3.1.2 The role of PAR_1 in thrombin induced-CCL2 production by mouse lung fibroblasts

Four PAR receptors (PAR_{1-4}) have been identified, and PAR_1, PAR_3, and PAR_4 are all activated by thrombin. In order to determine the role of PAR receptors for mediating the effect of thrombin on CCL2 production, the expression of PAR_{1-4} receptors in MLFs was first examined. Total RNA for MLFs was extracted using TRIzol, and quantitative real time RT-PCR (qRT-PCR) was performed for all four PARs. Values were normalized to 18S RNA and reported as a relative value by setting arbitrarily PAR_4 mRNA expression as 1. As shown in Figure 3.2, the expression of PAR_2 and PAR_3 was not detectable, whereas PAR_1 mRNA levels were 346 fold higher than PAR_4.

PAR_1 is the high-affinity thrombin receptor. It is cleaved at lower thrombin concentrations and preferentially cleaved when other PARs are present. The involvement of PAR_1 in thrombin-induced MLF CCL2 production was first examined. Serum-starved PAR_1 knockout MLFs (PAR_1 KO MLFs) were stimulated with 10 nM, 30 nM and 100 nM thrombin for 6 hours (Figure 3.3A). CCL2 protein levels in supernatants were assessed by ELISA. The results show that 10 nM thrombin did not induce CCL2 production in PAR_1 KO MLFs, suggesting that thrombin at 10 nM induces MLF CCL2 production via the activation of PAR_1. In contrast, a significant increase in CCL2 production was obtained with thrombin at concentrations of 30 nM and 100 nM (Figure 3.3A). It is well-known that thrombin can also signal via PAR_4 or via PAR-independent mechanism at relatively high concentrations (O'Brien *et al.*, 2001). In order to further investigate if PAR_4 mediates thrombin-induced CCL2 release in PAR_1 KO MLFs, the effects of PAR_4 agonist peptide AYPGKF and the reverse peptide YAPGKF were examined. Experiments were also performed with TNF-α as a positive control known to induce CCL2 independent of PAR signalling (Figure 3.3B). The results show that both AYPGKF and YAPGKF did not induce CCL2 release, whereas TNF-α induced a significant increase of CCL2 release. These data suggest that thrombin at concentrations higher than 10 nM induces MLF CCL2 production in a PAR-independent manner.

To further verify that 10 nM thrombin-induced CCL2 protein production was PAR_1-dependent, wild type and PAR_1 KO MLFs were exposed to thrombin (10 nM) and the specific PAR_1 agonist peptide TFLLR (200 μM) for 6 hours (Figure 4A). As shown in figure 3.4A, thrombin and TFLLR induced a 4.6 fold and an 8.2 fold increase respectively in CCL2 protein levels, by wild type MLFs (all $p<0.01$). In contrast, PAR_1 KO MLFs were completely unresponsive to both thrombin and TFLLR (Figure 3.4A). The effect of the inactive reverse peptide RLLFT on thrombin-induced CCL2 production was also examined in parallel cultures and the results show that RLLFT had no effect in both wild type and PAR_1 KO mouse lung fibroblasts. These data suggest that the effect obtained with TFLLR in MLFs is PAR_1-mediated.

In order to further confirm that thrombin-induced CCL2 production was PAR_1-dependent at 10 nM, the effect of the specific PAR_1 antagonist RWJ-58259 on thrombin-induced CCL2 production was investigated (Figure 3.4B). Cells were pre-incubated with increasing concentrations of RWJ-58259 for 15 minutes prior to stimulation with 10 nM thrombin for 6 hours. The results show that inhibition of PAR_1 by RWJ-58259 blocked CCL2 protein production in a concentration-dependent manner

from 0.3 µM (-Log [0.3 µM]=6.5) onwards. RWJ-58259 at 3 µM (-Log [3 µM]=5.5) inhibited this response by 95.6 ± 5.18% ($p<0.01$). The IC_{50} of RWJ-58259 was determined to be 0.4 µM for this response.

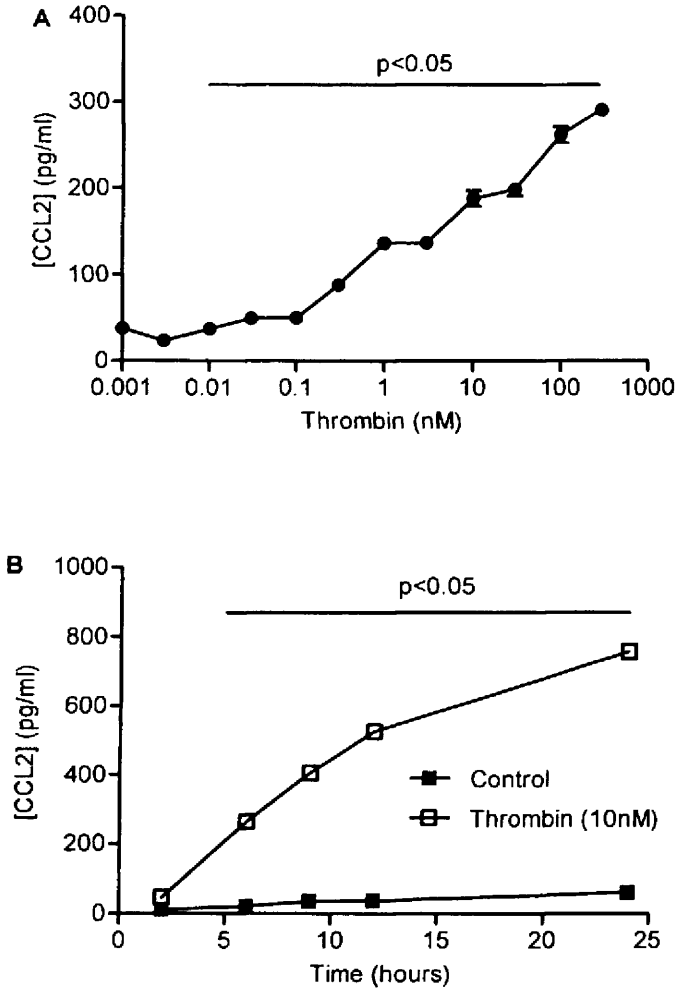


Figure 3.1: Thrombin stimulates MLF CCL2 protein production in a concentration- and time-dependent manner.

Panels A and B show concentration response (A) and time-course (B) data for the effect of thrombin on MLF CCL2 protein production. MLFs were exposed to thrombin (0.003 nM to 300 nM) for 6 hours, or exposed to thrombin (10 nM) for varying durations (2 to 24 hours). Supernatants from cell cultures following incubation were analyzed for CCL2 protein secretion by ELISA. The amount of secreted CCL2 is expressed as pg/ml and each value represents the mean ± SEM from triplicates at each concentration or time point. Data show the representative of three independent experiments. $p<0.05$ comparison with unstimulated cells or time point-matched media control.

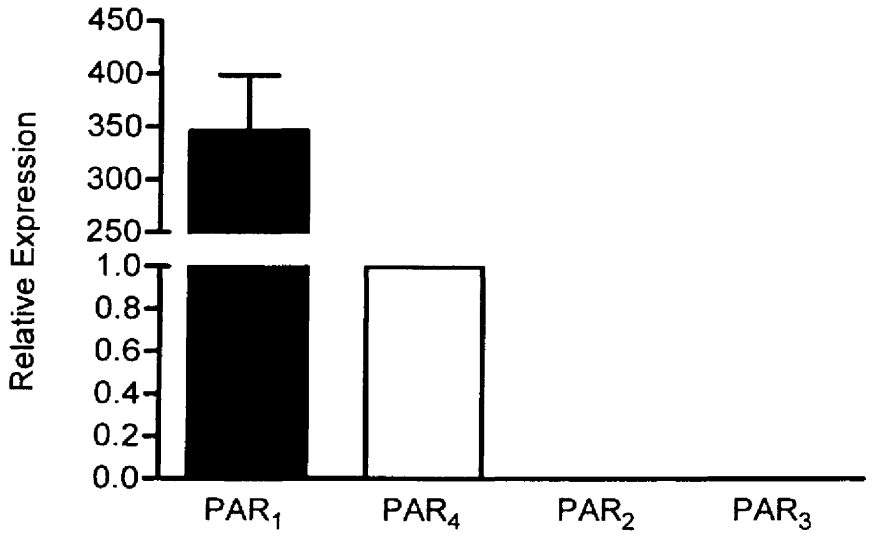


Figure 3.2: PAR gene expression in MLFs.

Figure shows the gene expression levels of PARs in MLFs. Total RNA for MLFs were exacted using TRIzol. Quantitative real-time RT-RCR (qRT-PCR) was performed using the Platinum SYBR green qPCR SuperMix UDG. Expression levels of PAR_1, PAR_2, PAR_3 and PAR_4 was normalized by 18S RNA and reported relative to PAR_4 expression. Data show the representative of three independent experiments.

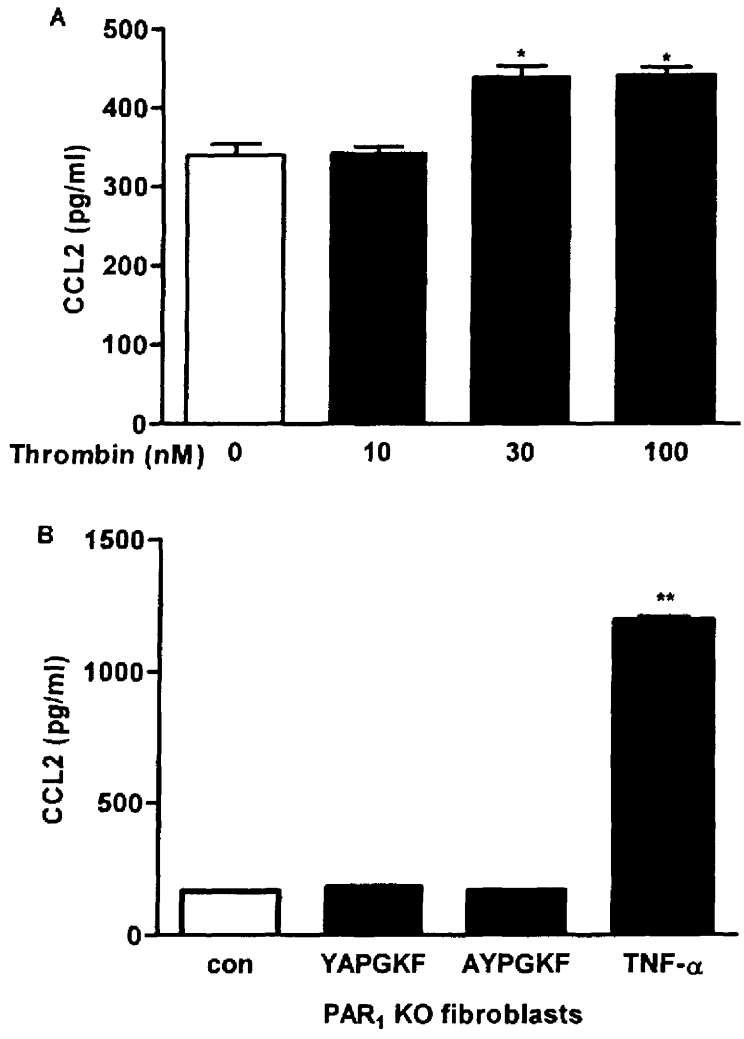


Figure 3.3 Thrombin at concentrations higher than 10 nM induces CCL2 production via a PAR-independent mechanism.

Panel A shows the concentration response effect of thrombin on PAR_1 KO MLF CCL2 protein production. PAR_1 KO MLFs were exposed to thrombin (10, 30 and 100 nM) for 6 hours. Panel B shows the effects of PAR_4 agonist AYPGKF, the reverse peptide YAPGKF and TNF-α on PAR_1 KO MLF CCL2 production. Cells were stimulated with AYPGKF, YAPGKF and TNF-α for 6 hours. CCL2 levels in culture supernatants were measured by ELISA. The amount of secreted CCL2 is expressed as pg/ml and each value represents the mean ± SEM from triplicates at each condition. Data show the representative of three independent experiments. *$p<0.05$, **$p<0.01$ comparison with unstimulated cells.

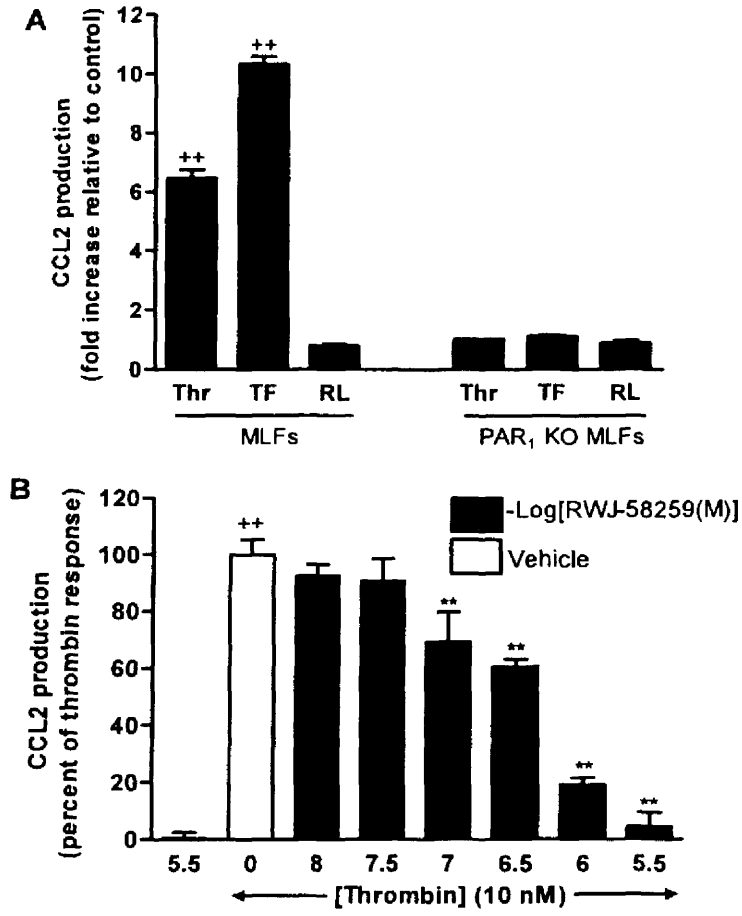


Figure 3.4 PAR_1 is necessary and sufficient for thrombin (10 nM) induced CCL2 protein production

Panel A shows the effects of thrombin, PAR_1 agonist TFLLR-NH_2, and the reverse peptide RLLFT-NH_2 on CCL2 protein production in MLFs and PAR_1 KO fibroblasts. Cells were exposed to thrombin (Thr, 10 nM), TFLLR (TF, 200 μM) or RLLFT (RL, 200 μM) for 6 hours. Data are presented as fold-increase relative to media control. Panel B shows effect of the PAR_1 antagonist RWJ-58259 on MLF CCL2 protein production in response to thrombin. Data are presented as a percentage of the maximal response obtained with thrombin with drug vehicle alone (0.03% DMSO in DMEM). Cells were treated with increasing concentrations of RWJ-58259 for 15 minutes before stimulation with thrombin for 6 hours. Final concentrations of DMSO were kept constant for all treatment conditions. The first bar represents the highest concentration of RWJ-58259 and shows that this compound has no effect on basal CCL2 production. Negative log of the concentrations of RWJ-58259 are presented. Data represent the mean ± SEM of triplicates. Data show the representative of three independent experiments. CCL2 levels in culture supernatants were measured by ELISA. $^{++}p<0.01$ comparison with unstimulated cells; $^{**}p<0.01$ comparison with thrombin alone.

3.1.3 Effect of thrombin on CCL2 mRNA levels in mouse lung fibroblasts

The data obtained so far strongly indicate that PAR_1 is necessary and sufficient for thrombin-induced MLF CCL2 protein release at 10 nM. To determine whether thrombin could similarly influence CCL2 gene expression, the effect of thrombin on CCL2 mRNA levels was assessed by qRT-PCR. As shown in figure 3.5 A, thrombin increased CCL2 mRNA levels significantly within 30 minutes, with a maximal increase (21 ± 2.9 fold relative to control) observed at 6 hours (Figure 3.5A, $p<0.01$). CCL2 mRNA levels declined after 9 hours and returned to baseline level at 24 hours. To determine if thrombin induces CCL2 protein release transcriptionally or post-transcriptionally, the effect of actinomycin-D (act-D: inhibitor of transcription) and cycloheximide (CHX: inhibitor of protein synthesis) on CCL2 mRNA levels induced by thrombin was next examined. MLFs were pre-incubated with act-D (1 μg/ml) for 30 minutes, or pre-incubated with CHX (1 μM) for 1 hour, and subsequently stimulated with 10 nM thrombin for 2 hours (Figure 3.5 B&C). As shown in figure 3.5B, act-D almost completely abolished thrombin-induced CCL2 mRNA levels (88.27 ± 0.037%), suggesting that thrombin-induced CCL2 protein release is not due to the release of prestored CCL2. In contrast, experiments performed in the presence of CHX (Figure 3.5C) showed that CHX alone significantly induced CCL2 mRNA levels, and that thrombin-induced CCL2 mRNA expression was also dramatically enhanced by CHX. This suggests that CCL2 mRNA levels are highly regulated by a labile repressor at baseline, which may not be overcome by the addition of thrombin.

3.1.4 Effect of PAR_1 on thrombin-induced CCL2 mRNA levels

To determine whether PAR_1 mediates thrombin-induced CCL2 production via the increase of CCL2 mRNA level, the influence of the specific PAR_1 antagonist RWJ-58259 on thrombin-induced CCL2 mRNA levels was investigated (Figure 3.6). Pre-incubation of MLFs with RWJ-58259 (1 μm) for 15 minutes prior to stimulation with 10 nM thrombin for 2 hours almost completely abolished this response, indicating that PAR_1 is necessary for thrombin-induced CCL2 mRNA levels, and PAR_1 mediates thrombin-induced CCL2 production via a transcriptional mechanism.

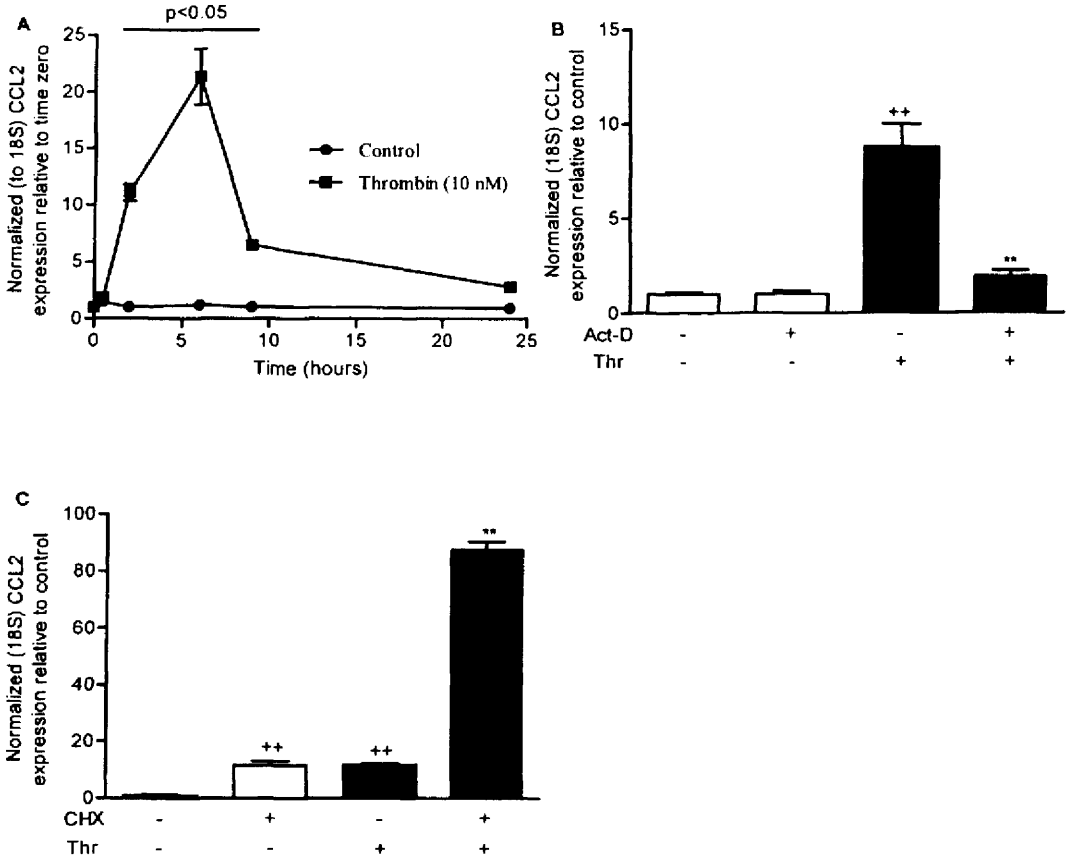


Figure 3.5 Thrombin increases CCL2 protein via increased gene expression in MLFs.
Panel A shows time-course data for the effect of thrombin on CCL2 mRNA levels. MLFs were exposed to serum-free control medium (DMEM) or thrombin (10 nM) for varying duration from 0.5 to 24 hours. CCL2 mRNA levels at each time point were assessed by qRT-PCR. Data are expressed as fold change relative to time zero for each time point (mean ± SEM from triplicates) following normalization to 18S RNA. Panel B shows the effect of act-D on thrombin-induced CCL2 mRNA levels. MLFs were exposed to thrombin for 2 hours with or without pre-incubation with act-D (1 μg/ml) for 30 minutes. CCL2 mRNA levels were determined as in panel A. act-D was dissolved in ethanol and the same amount of ethanol was added for all cultures (0.1% ethanol in DMEM). Panel C shows the effect of CHX on thrombin-induced CCL2 mRNA levels. MLFs were exposed to thrombin for 2 hours with or without pre-incubation with CHX (1 μM) for 1 hour. CCL2 mRNA levels were determined as in panel A. Data represent mean ± SEM from triplicates at each condition, and show the representative of three independent experiments. $p<0.05$, comparison with time-point matched medium control; $^{++}p<0.01$, comparison with unstimulated cells; $^{**}p<0.01$, comparison with thrombin alone.

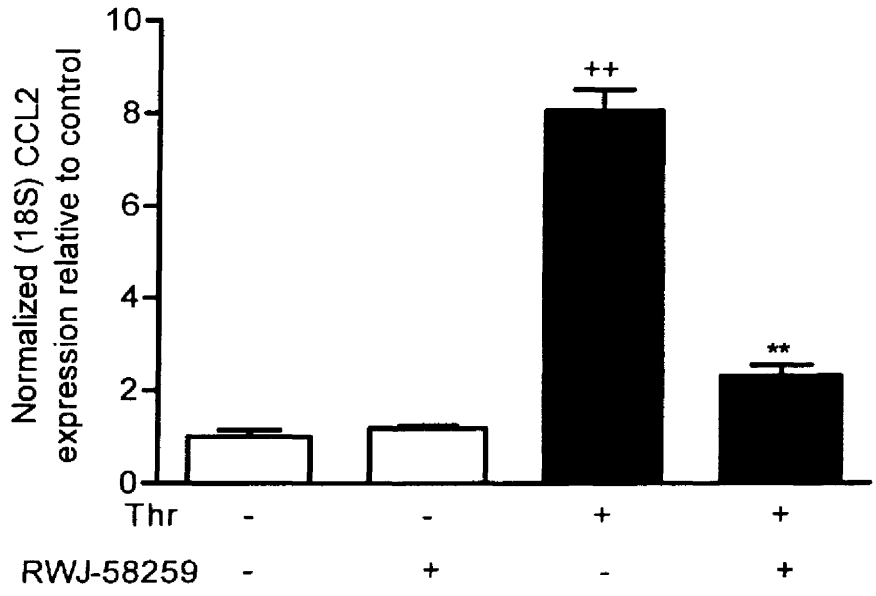


Figure 3.6 PAR_1 mediates thrombin-induced MLF CCL2 mRNA levels.

Figure shows the effect of RWJ-58259 on thrombin-induced MLF CCL2 mRNA levels. MLFs were exposed to thrombin for 2 hours with or without pre-incubation with RWJ-58259 (1 μM) for 15 minutes. CCL2 mRNA levels at each condition were assessed by qRT-PCR. Data are expressed as fold change relative to untreated group (mean ± SEM from triplicates) following normalization to 18S RNA. Data show the representative of three independent experiments.

3.1.5 Summary

The results described in this section examining the effect of thrombin on MLF CCL2 protein release and mRNA levels showed that:

- Thrombin induces MLF CCL2 protein release in a time and concentration-dependent manner from 0.03 nM onwards.
- PAR_1 is strongly expressed in MLFs; PAR_2 and PAR_3 are not detectable, whereas PAR_4 is weakly expressed.
- PAR_1 is necessary and sufficient for mediating thrombin (10 nM)-induced CCL2 production in MLFs.
- The effect of thrombin on MLF CCL2 release at higher concentrations (> 10 nM) is likely to be independent of PAR signalling.
- Thrombin induced MLF CCL2 mRNA levels in a time-dependent manner and this is mediated by PAR_1 since inhibition of PAR_1 by RWJ-58259 abolished this response.
- Gene transcription is an important mechanism leading to the enhanced CCL2 production in MLFs in response to thrombin as the transcription inhibitor act-D completely abolished thrombin-induced CCL2 mRNA levels.
- *De novo* protein synthesis is not required for thrombin-induced CCL2 mRNA levels since the protein synthesis inhibitor CHX did not inhibit this response.

In summary, thrombin (10 nM) induces CCL2 protein release and mRNA levels via the activation of PAR_1, and gene transcription is an important mechanism leading to the enhanced CCL2 protein production.

3.2 The role of G proteins in thrombin-induced CCL2 mRNA levels and protein production

In order to begin to unravel the mechanisms by which thrombin exerts its stimulatory effects on CCL2 mRNA and protein levels, I first examined the potential involvement of G proteins, since PAR_1 exerts its pluripotent cellular effects via the ability to interact with multiple downstream G proteins, including $G\alpha_{i/o}$, $G\alpha_{q/11}$ and $G\alpha_{12/13}$.

3.2.1 Effect of pertussis toxin (PTX) on PAR_1-dependent CCL2 production

To evaluate whether PAR_1-mediated CCL2 production is $G\alpha_i$-dependent, the effect of $G\alpha_i$ specific inhibitor PTX on this response was examined. MLFs were pre-incubated with PTX (100 ng/ml) for 6 hours prior to exposure to thrombin (10 nM) for 6 hours (Figure 3.7A). The data show that PTX increased the basal and thrombin-induced CCL2 production. This finding suggests that a $G\alpha_i$ protein-coupled pathway exerts negative regulatory control of CCL2 production, and thrombin-induced CCL2 production is independent of PAR_1 coupling to $G\alpha_i$ and its associated downstream signalling pathways. In order to verify the complete ADP-ribosylation of $G\alpha_i$ after pretreatment of MLFs with PTX, untreated and PTX-treated (100 ng/ml; for 6 hours) MLFs were lysed, and lysates were analyzed by western blotting with an antibody against $G\alpha_i$ (Figure 3.7B). Complete ADP-ribosylation after PTX treatment is indicated by the decreased mobility of $G\alpha_i$-type G-proteins in SDS-polyacrylamide gels. This data further confirm that the lack of an inhibitory effect of PTX on thrombin-induced CCL2 release is not due to the incomplete inhibition of $G\alpha_i$ activity.

3.2.2 PAR_1 coupling to $G\alpha_q$ is necessary for PAR_1-mediated CCL2 protein release and CCL2 mRNA levels

As PAR_1 can also interact with $G\alpha_q$ and $G\alpha_{12/13}$, the next experiments performed were to evaluate whether thrombin-induced CCL2 protein production was dependent on PAR_1 coupling to $G\alpha_q$ and/or $G\alpha_{12/13}$. A genetic inhibition approach was employed to address this question. MLFs were transduced with retroviral constructs encoding EGFP or $G\alpha_q$, $G\alpha_i$ and $G\alpha_{12/13}$ minigenes. These G protein minigenes encode 11 unique carboxyl-terminal amino acid residues of Gα subunits and have previously been shown to effectively inhibit G protein signalling, including thrombin-mediated cellular effects (Ellis *et al.*, 1999; Gilchrist *et al.*, 2001; Vanhauwe *et al.*, 2002). Transfection efficiency was monitored by visualizing EGFP expression in control cells and approximately 90% of EGFP-transduced cells were EGFP positive as shown in Figure 3.8 A&B. Transduced-MLFs were then exposed to thrombin for 6 hours. The supernatants were analyzed for CCL2 production by ELISA. As shown in figure 3.8C, the $G\alpha_q$ C-terminal

antagonist encoding minigene completely abolished thrombin-induced CCL2 release. In contrast, transduction with a control minigene encoding EGFP, or C-terminal $G\alpha_i$, $G\alpha_{12}$ or $G\alpha_{13}$ had no effect on this response, suggesting that subsequent activation of $G\alpha_q$ by PAR_1 mediates thrombin-induced CCL2 production in MLFs, whereas $G\alpha_i$ and $G\alpha_{12/13}$ are not involved.

In order to further confirm the role of $G\alpha_q$ in mediating PAR_1 activation-induced CCL2 production, I next examined the effect of a novel small molecule antagonist, Q94, on this response. Q94 is a small molecule that specifically targets PAR_1 coupling to $G\alpha_q$. Figure 3.9A shows that Q94 blocked PAR_1-mediated CCL2 production in a concentration-dependent manner with complete inhibition obtained at 10 μM. Furthermore, inhibition of PAR_1 coupling to $G\alpha_q$ with Q94 (10 μM) also completely abolished PAR_1-specific agonist peptide TFLLR-induced CCL2 production (Figure 3.9B), further suggesting that PAR_1 coupling to $G\alpha_q$ is necessary and sufficient for mediating thrombin-induced CCL2 production. In order to evaluate the specificity of Q94, the effect of Q94 on TNF-α-induced CCL2 production in MLFs was examined (Figure 3.9C). Experiments performed with TNF-α as the stimulus showed that Q94 (3 and 10 μM) had no effect on TNF-α-stimulated CCL2 production, indicating that the antagonist was specific for PAR_1 in blocking this response.

To evaluate whether PAR_1-coupling to $G\alpha_q$ mediates thrombin-induced CCL2 production via increased CCL2 mRNA levels, the role of $G\alpha_q$ in thrombin-induced CCL2 mRNA levels was next examined. MLFs were pre-incubated with Q94 (10 μM) for 3 hours before exposure to thrombin for 2 hours. The subsequent mRNA levels were assessed by qRT-PCR. As shown in figure 3.10, Q94 significantly blocked thrombin-induced CCL2 mRNA levels by about 70% ($p<0.01$).

Taken together, these data show that PAR_1 coupling to $G\alpha_q$ plays a central role in mediating thrombin-induced CCL2 protein production and mRNA levels.

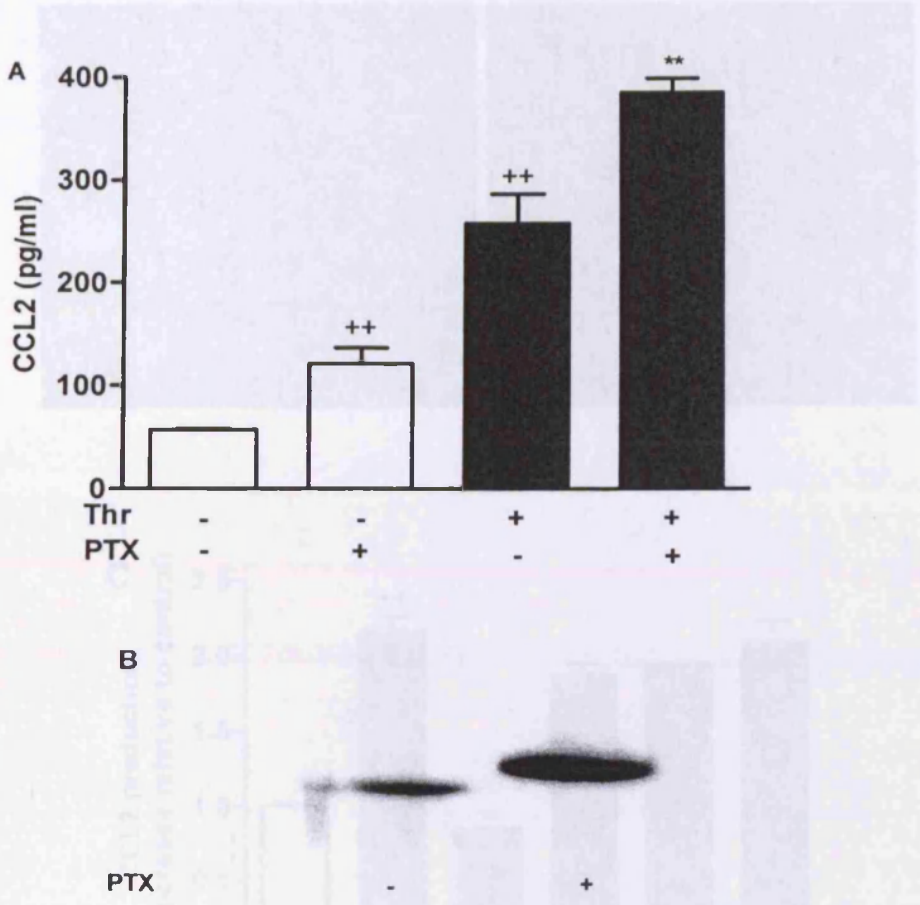


Figure 3.7 Pertussis toxin (PTX) increases basal and thrombin-induced CCL2 protein production.

Panel A shows the effect of $G_{i/o}$ inhibition by PTX on basal and thrombin-induced CCL2 production. Cells were pre-incubated with PTX (100 ng/ml) for 6 hours before being exposed to thrombin (10 nM) for 6 hours. Supernatants from cell cultures following incubation were analyzed for CCL2 protein secretion by ELISA. Data represent the mean ± SEM of triplicates. $^{++}p<0.01$, comparison with medium control in the absence of PTX; $^{**}p<0.01$, comparison with thrombin alone. Panel B shows the complete ADP-ribosylation of $G\alpha_i$ after pretreatment of cells with PTX. Cells were treated with or without PTX (100 ng/ml) for 6 hours. Cell lysates were analyzed by western blotting with an antibody against $G\alpha_i$. Data show the representative of three independent experiments.

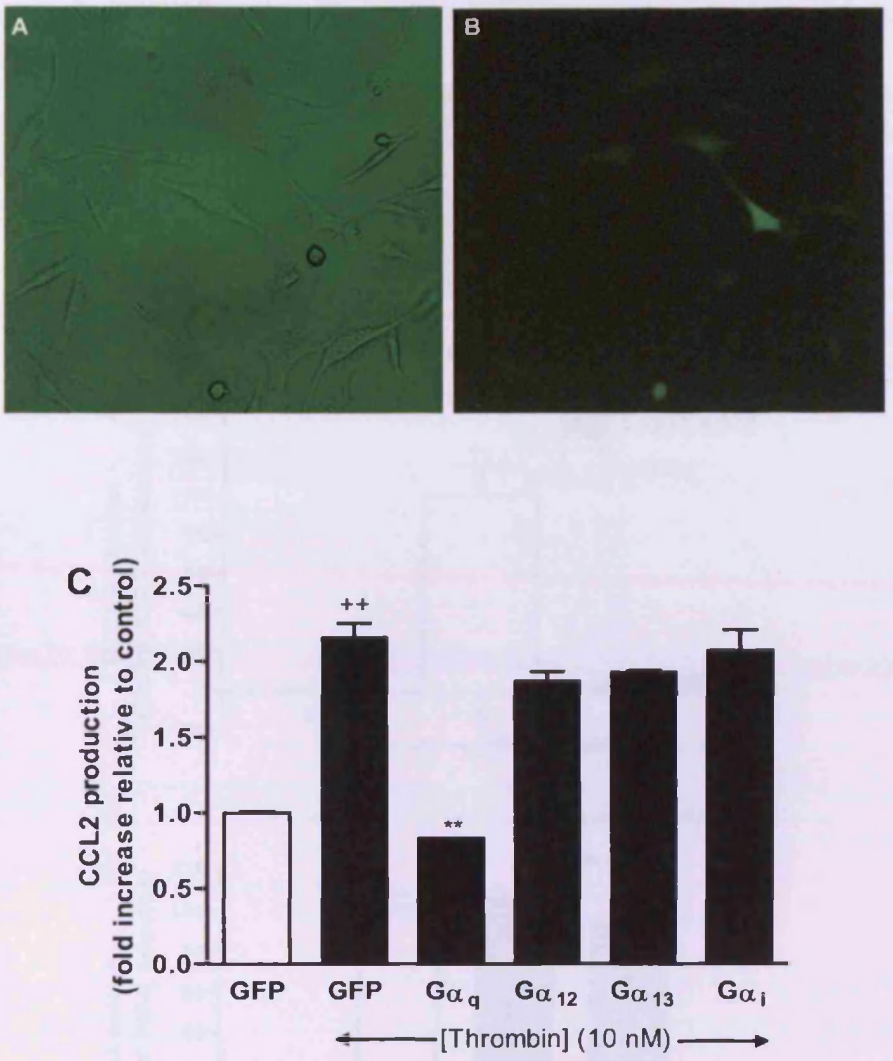


Figure 3.8 $G\alpha_q$ is necessary for mediating thrombin-induced CCL2 protein production. Panel A and B show the representative analysis for MLF positive for enhanced green fluorescent protein (EGFP), showing the efficient transduction of the plasmid vector carrying GFP gene. Panel B shows the effects of G protein minigenes on thrombin-induced CCL2 protein production. MLFs transduced with the pRevTRE2-EGFP, pRevTRE2-$G\alpha_q$, pRevTRE2-$G\alpha_{12}$, pRevTRE2-$G\alpha_{13}$, or pRevTRE2-$G\alpha_i$ were exposed to thrombin (10 nM) for 6 hours and CCL2 levels in culture supernatant were measured by ELISA. Data are expressed as fold increase of CCL2 production relative to GFP control. Data show the representative of three independent experiments. $^{++}p<0.01$, comparison with medium control; $^{**}p<0.01$, comparison with thrombin alone.

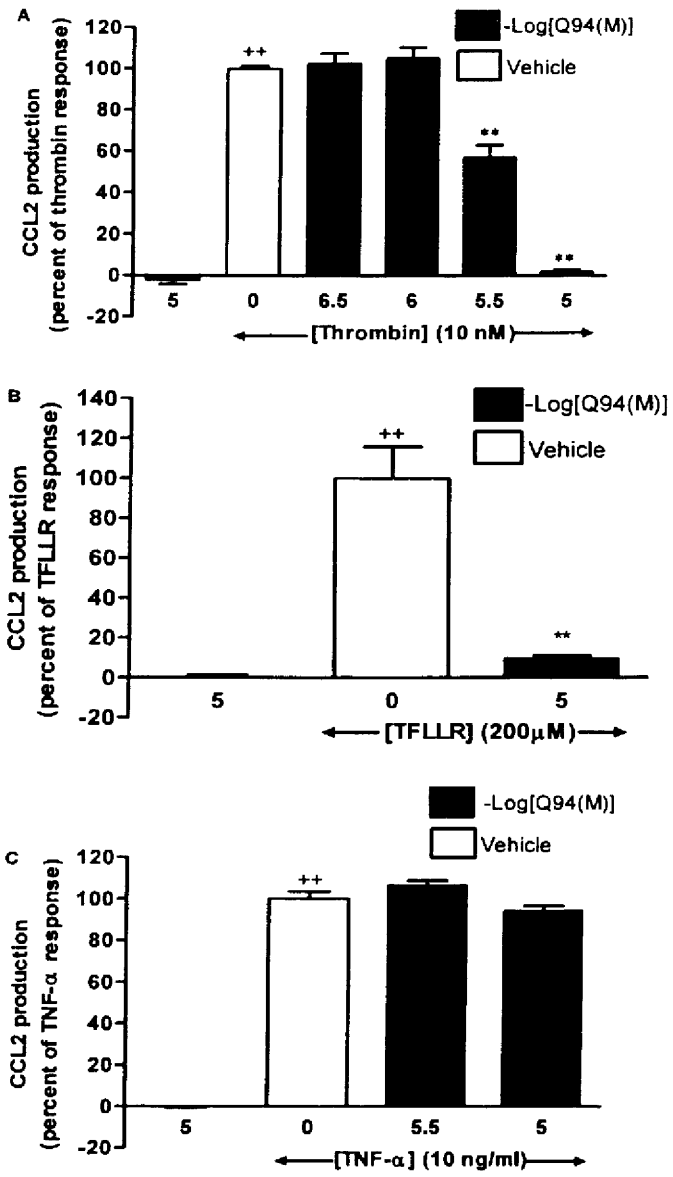


Figure 3.9 PAR_1 coupling to $G\alpha_q$ is necessary and sufficient for thrombin-induced CCL2 protein production.

Panel A, B and C show the effects of antagonist Q94 on thrombin (A, 10 nM), TFLLR (B, 200 μM) and TNF-α (C, 10 ng/ml)-induced CCL2 production. Data are presented as a percentage of the maximal response obtained with thrombin, TFLLR, TNF-α and drug vehicle alone (0.1% DMSO in DMEM). Cells were treated with increasing concentrations of Q94 for 3 hours before exposure to thrombin, TFLLR or TNF-α for 6 hours. Final concentrations of DMSO were kept constant for all treatment conditions. The first bars represent the highest concentration of Q94 used and show that this compound has no effect on basal CCL2 production in three experiments. Negative log of the concentrations of Q94 are presented. Data show the

representative of three independent experiments. ++ p<0.01, comparison with medium control; ** p<0.01, comparison with thrombin, TFLLR or TNF-α alone.

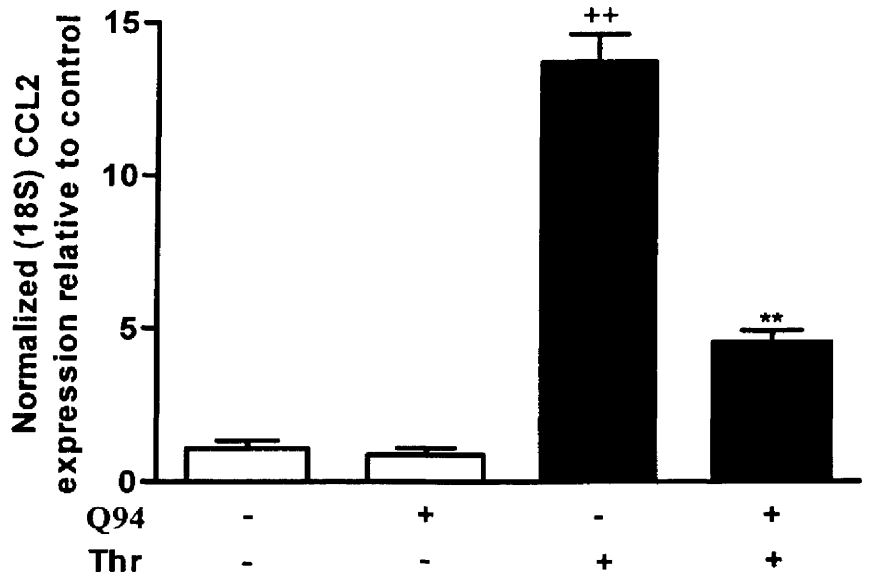


Figure 3.10 PAR_1 coupling to $G\alpha_q$ is necessary for thrombin-induced MLF CCL2 mRNA levels.

Figure shows the effect of Q94 on thrombin-induced CCL2 mRNA levels. MLFs were exposed to thrombin for 2 hours with or without pre-incubation with Q94 (10 μM) for 3 hours. Data represent the mean ± SEM of triplicates, and show the representative of three independent experiments. ++ p<0.01, comparison with medium control; ** p<0.01, comparison with thrombin alone.

3.2.3 Summary

The results described in this section examining the role of G protein α subunits in PAR_1-induced CCL2 protein release and mRNA levels showed that:

- Thrombin-induced CCL2 production in MLFs is not dependent on PAR_1 coupling to $G\alpha_i$ as inhibition of $G\alpha_i$ by both PTX and $G\alpha_i$ minigene did not inhibit this response.

- $G\alpha_{12}$ and $G\alpha_{13}$ are not involved in mediating thrombin-induced CCL2 production since inhibition of both $G\alpha_{12}$ by $G\alpha_{12}$ minigene and $G\alpha_{13}$ by $G\alpha_{13}$ minigene did not block this response of thrombin.

- PAR_1 coupling to $G\alpha_q$ is necessary for mediating thrombin-induced CCL2 production since this effect of thrombin was completely abolished by both $G\alpha_q$ minigene and the $G\alpha_q$ selective PAR_1 antagonist Q94.

- PAR_1 coupling to $G\alpha_q$ is also sufficient for mediating thrombin-induced CCL2 production as Q94 abolished the CCL2 production induced by PAR_1-specific agonist peptide TFLLR.

- PAR_1 coupling to $G\alpha_q$ mediates thrombin-induced CCL2 protein release via increased CCL2 mRNA levels since inhibition of PAR_1 coupling to $G\alpha_q$ by Q94 significantly inhibited thrombin-induced CCL2 mRNA levels.

In summary, thrombin-induced CCL2 protein release and mRNA levels are mediated by PAR_1 coupling to $G\alpha_q$.

3.3 The role of MAP kinases in thrombin-induced MLF CCL2 production

Thrombin is known to exert its numerous cellular effects in fibroblasts via MAP kinase (p38, JNK and ERK1/2 kinases) pathways (Bogatkevich *et al.*, 2005; Wang *et al.*, 2006), and previous studies performed in MLFs have shown that activation of $G\alpha_q$ by PAR_1 leads to the activation of ERK1/2 (Trejo *et al.*, 1996). However, in terms of thrombin-induced CCL2 release by other cell types, the p38 MAPK pathway has been shown to play an important role (Brandes *et al.*, 2001; Marin *et al.*, 2001). Thus, experiments were performed next to determine the relative roles of these MAPK kinases in PAR_1-mediated CCL2 production.

3.3.1 Effect of MAP kinase inhibition on thrombin-induced MLF CCL2 production

The role of p38 in thrombin-induced MLF CCL2 production was first examined. Cells were pre-incubated with increasing concentrations of the p38 specific inhibitor SB203580 for 30 minutes prior to stimulation with thrombin (10 nM) for 6 hours. As shown in figure 3.11A, inhibition of p38 MAPK pathway with SB203580 had no effect on basal or thrombin-induced CCL2 production. In order to verify the specificity of SB203580, the effect of its inactive control SB202474 on thrombin-induced CCL2 production was examined (Figure 3.11B). Surprisingly, the results show that SB202474 at the highest concentration used significantly inhibited thrombin-induced CCL2 release by ~50%, suggesting that at the high concentrations these compounds may exert off-target effects. To further exclude the involvement of p38 in thrombin-induced CCL2 release, thrombin-induced p38 phosphorylation was examined. MLFs were stimulated with thrombin over a period ranging from 30 seconds to 2 hours (Figure 3.12A). P38 phosphorylation was examined by western blotting using a phospho-p38 specific antibody. Time-course data showed that thrombin induced p38 phosphorylation in 2 minutes and phosphorylated p38 was undetectable in un-stimulated cells. The signal faded away after 2 minutes and diminished after 1 hour stimulation. The effect of SB203580 on thrombin-induced p38 phosphorylation was next examined. Cells pre-incubated with SB203580 (2 μM) for 30 minutes were exposed to thrombin for 2 minutes, cell lysate were analyzed by western blotting. The results show that SB203580 at highest concentration used completely inhibited thrombin-induced p38 phosphorylation. It is therefore concluded that although thrombin activated p38 MAPK in MLFs, p38 is not involved in mediating PAR_1-induced CCL2 release.

The role of JNK was next examined. MLFs were pre-incubated with JNK inhibitor TI-JIP for 30 minutes before exposed to thrombin for 6 hours (Figure 3.13). Surprisingly, the

data show that inhibition of JNK pathway by TI-JIP did not block thrombin-induced CCL2 production, but rather it increased both basal and thrombin-stimulated CCL2 protein release in a concentration-dependent manner from 1 μM onwards (all $p<0.01$) (Figure 3.13). These data suggest that JNK pathway did not mediate thrombin-induced CCL2 production.

Conversely, preincubation of MLFs with the MEK1/2 inhibitor U0126 blocked thrombin-induced CCL2 production in a concentration-dependent manner from 0.3 μM (-Log [0.3 μM]=6.5) onwards (Figure 3.14A). At 10 μM (-Log [10 μM]=5), U0126 inhibited thrombin-induced CCL2 production by 84.7±0.26% ($p<0.01$). The IC_{50} of U0126 was determined to be 1.3 μM for this response. As MEK1/2 is a direct upstream activator of ERK1/2, the inhibitory effect obtained with U0126 infers the involvement of ERK1/2 in PAR_1-mediated CCL2 production. To further strengthen the results obtained with U0126 and to further confirm the involvement of ERK1/2 in this response, another widely used, but structurally unrelated MEK1/2 inhibitor PD98059 was also employed and the effect of PD98059 on thrombin-induced CCL2 production was also examined. As shown in figure 3.14B, PD98059 at the commonly used concentration (50 μM) also inhibited PAR_1-mediated CCL2 production, suggesting that MEK1/2 by referring to ERK1/2 is involved in PAR_1-mediated CCL2 production.

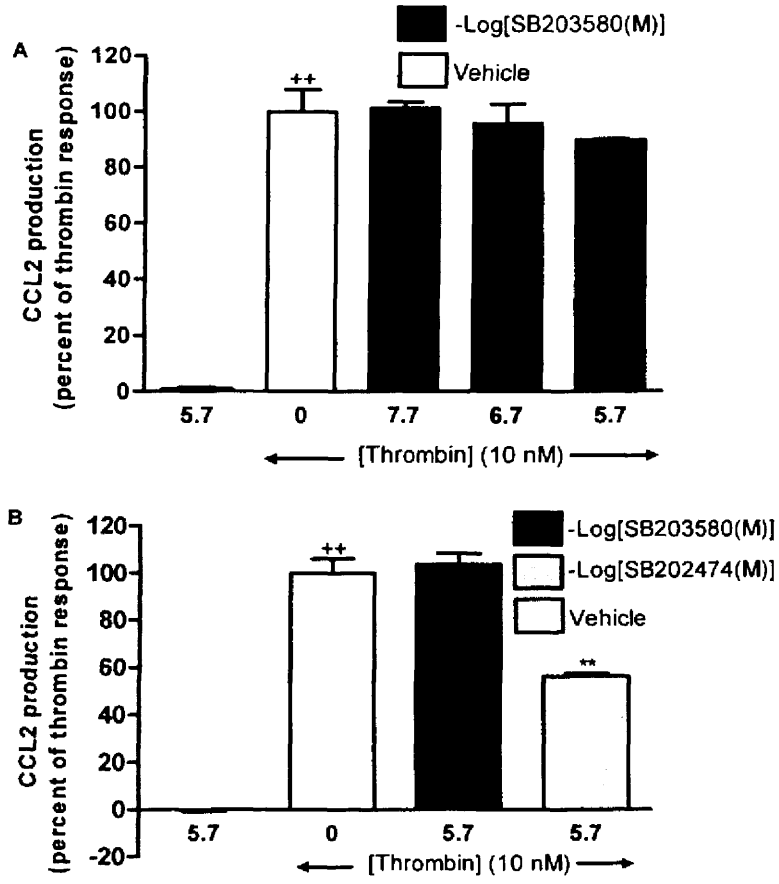


Figure 3.11 Thrombin-induced CCL2 production by MLFs and this effect is not blocked by p38 kinase inhibition.

Panel A and B show the effects of SB203580 and SB204797 on CCL2 protein production in response to thrombin. Data are presented as a percentage of the maximal response obtained with thrombin and drug vehicle alone (0.02% DMSO in DMEM). Cells were treated with increasing concentrations of SB203580 or SB 204797 for 30 minutes before exposure to thrombin (10 nM) for 6 hours. Final concentrations of DMSO were kept constant for all treatment conditions. The first bars represent the highest concentration of inhibitors used, and show that these compounds have no effect on basal CCL2 production. Negative log of the concentrations of each inhibitor are presented. Data show the representative of three independent experiments. $^{++}$ $p<0.01$, comparison with unstimulated cells; ** $p<0.01$, comparison with thrombin alone.

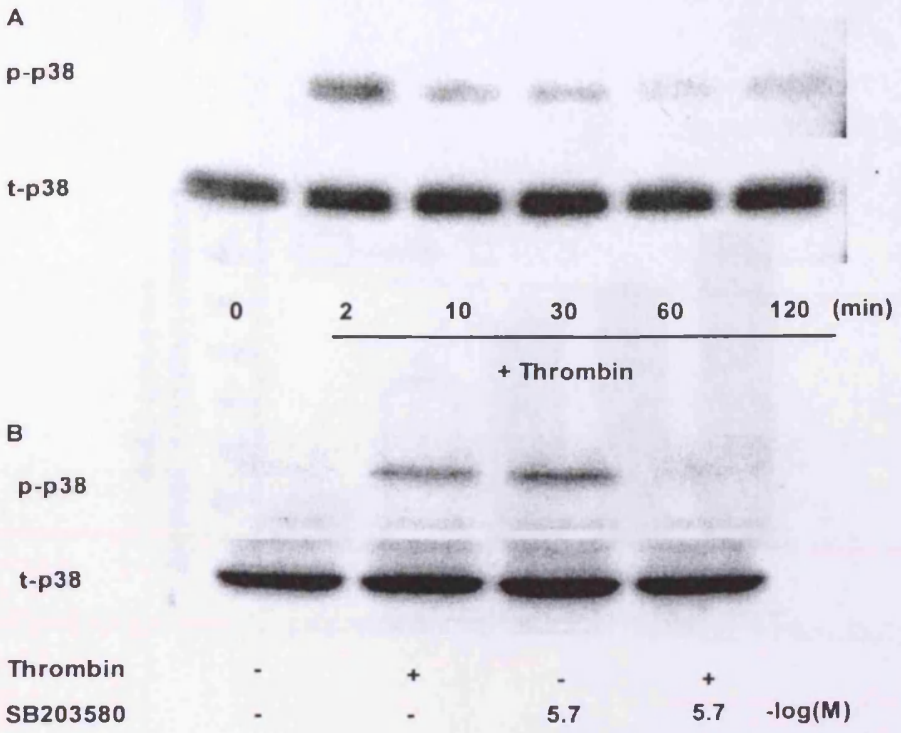


Figure 3.12 Thrombin induces p38 phosphorylation in a time-dependent manner and this effect is inhibited by SB203580 in MLFs.

Panel A shows that thrombin induced p38 phosphorylation in a time-dependent manner. MLFs were exposed to thrombin (10 nM) for the indicated time periods. P38 phosphorylation was assessed by western blotting using an anti phospho-p38 antibody. Protein loading control was verified by blotting with an anti-total p38 antibody (lower panel). Panel B shows the effect of SB203580 on thrombin-induced p38 phosphorylation. Cells were pre-incubated with p38 (2 μM) for 30 minutes followed by thrombin stimulation for 2 minutes. P38 phosphorylation was assessed by western blotting using an anti phosphor-p38 antibody (upper panel). Protein loading control was verified by blotting with a total p38 antibody (lower panel). Data show the representative of three independent experiments.

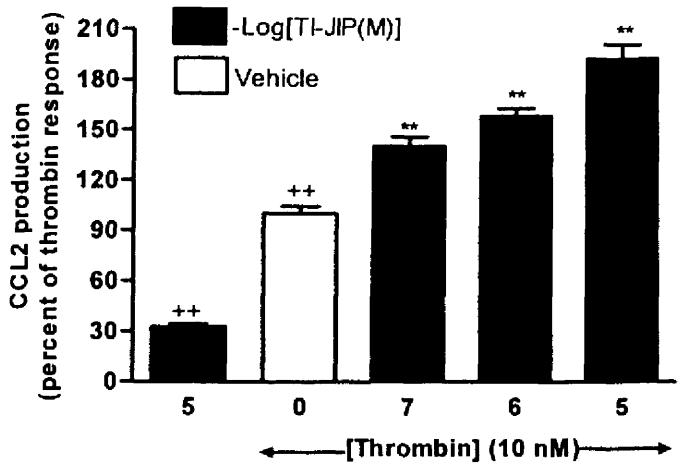


Figure 3.13 Thrombin-induced CCL2 production by MLFs is not attenuated by JNK inhibition.

Figure shows the effect of TI-JIP on CCL2 protein production in response to thrombin. Data are presented as a percentage of the maximal response obtained with thrombin and drug vehicle alone (0.1% DMSO in DMEM). Cells were treated with increasing concentrations of TI-JIP for 30 minutes before exposure to thrombin (10 nM) for 6 hours. Final concentrations of DMSO were kept constant for all treatment conditions. The first bar represents the highest concentration of TI-JIP examined, and shows that this compound increases the basal CCL2 production. Negative log of the concentrations of each inhibitor are presented. Data show the representative of three independent experiments. $^{++}$ $p<0.01$, comparison with untreated cells; ** $p<0.01$, comparison with thrombin alone.

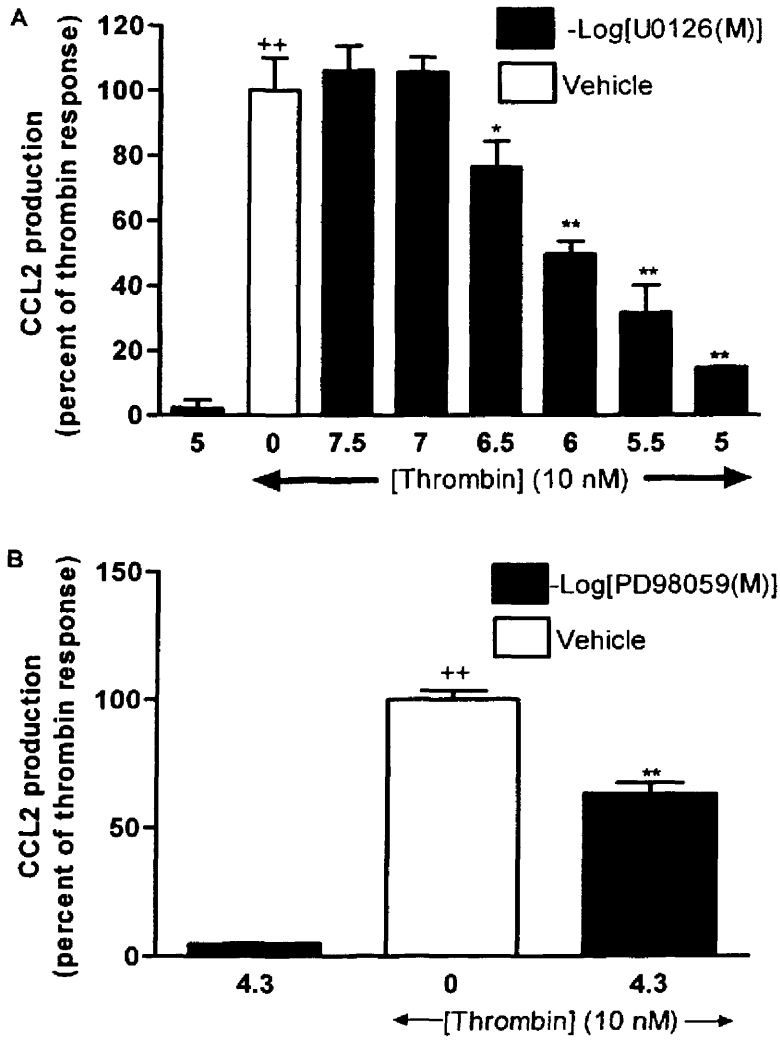


Figure 3.14 Thrombin-induced CCL2 production is attenuated by MEK1/2 inhibition.
Panel A and panel B show the effects of U0126 (A) and PD98059 (B) on CCL2 protein production in response to thrombin (10 nM). Data are presented as a percentage of the maximal response obtained with thrombin with drug vehicle alone (0.1% DMSO in DMEM). Cells were treated with increasing concentrations of U0126, or PD98059 for 30 minutes before stimulation with thrombin for 6 hours. Final concentrations of DMSO Were kept constant for all treatment conditions. The first bars represent the highest concentration of inhibitors used and show that these compounds have no effect on basal CCL2 production. Negative log of the concentrations of U0126 are presented. Data represent the mean ± SEM of triplicates, and show the representative of three independent experiments.. $^{++}p<0.01$, comparison with unstimulated cells; $^{*}p<0.05$ and $^{**}p<0.01$, comparison with thrombin alone.

3.3.2 Further experimental evidence for the involvement of ERK1/2 in PAR_1-mediated CCL2 production

3.3.2.1 Thrombin induces endogenous ERK1/2 phosphorylation: a time course

To further investigate the involvement of ERK1/2 in PAR_1-mediated CCL2 production, thrombin-induced ERK1/2 phosphorylation was first examined. MLFs were challenged with thrombin (10 nM) over a period ranging from 30 seconds to 30 minutes (Figure 3.15A). ERK1/2 phosphorylation was examined by western blotting using a phospho-ERK1/2 specific antibody. The amount of phosphorylated ERK1/2 induced by thrombin in MLFs was normalized against the total amount of ERK1/2 (lower panel) and determined by densitometry (Figure 3.15B). As shown in figure 3.15A, thrombin induced ERK1/2 phosphorylation in a time-dependent manner in MLFs. Phosphorylated ERK1/2 was undetectable in un-stimulated cells (upper panel, lane 1). Thrombin induced strong phosphorylation of ERK1/2 as early as 30 seconds (upper panel, lane 2), with a maximal activation obtained at 2 minutes (upper panel, lane 3). The signal decreased after 5 minutes (upper panel, lane 4), and no ERK1/2 phosphorylation was seen after 30 minutes (upper panel, lane 6).

3.3.2.2 Effects of PAR_1 antagonist (RWJ-58259), $G\alpha_q$ selective PAR_1 antagonist (Q94) and MEK1/2 inhibitor (U0126) on thrombin-induced ERK1/2 phosphorylation

In order to determine whether ERK1/2 pathway is downstream of PAR_1 and PAR_1 coupling to $G\alpha_q$, the effects of a potent and selective PAR_1 antagonist RWJ-58259, and the $G\alpha_q$ selective PAR_1 antagonist Q94 on thrombin-induced ERK1/2 phosphorylation were examined (Figure 3.16). As shown in figure 3.16 A&B, pretreatment of MLFs with RWJ-58259 (1 μM) for 15 minutes completely abolished thrombin-induced ERK1/2 phosphorylation at 2 minutes. Pretreatment with Q94 (10 μM, Figure 3.16C) for 3 hours also completely blocked this response of thrombin, suggesting that ERK1/2 pathway is downstream of PAR_1 coupling to $G\alpha_q$.

To determine whether MEK1/2 is upstream of ERK1/2 in the pathway leading to CCL2 production, the effect of U0126 on thrombin-induced ERK1/2 phosphorylation was examined. The data show that U0126 significantly blocked thrombin-induced ERK1/2 phosphorylation in a concentration-dependent manner from 1 μM onwards (Figure 3.17). U0126 at 1 μM blocked ERK1/2 phosphorylation by 100%. These data demonstrate that PAR_1 and MEK1/2 act at upstream of ERK1/2 in the signalling pathway leading to CCL2 production by thrombin.

3.3.2.3 ERK1/2 is necessary and sufficient for CCL2 production induced by thrombin

To further confirm the role of ERK1/2 pathway in PAR_1-induced CCL2 production, a genetic approach was used. As MEK1 is the upstream regulator of ERK1/2, wild type MEK1 (wt-MEK1), dominant negative MEK1 (dn-MEK1) or constitutively active MEK1 (ca-MEK1) were transduced into MLFs. Figure 3.18A shows that thrombin-induced ERK1/2 phosphorylation was blocked by up to 70% in cells transduced with dn-MEK1. Figure 3.18B shows that ca-MEK1 significantly increased basal ERK1/2 phsophorylation, but had no additive effect on thrombin-induced ERK1/2 phosphorylation. Transduced-MLFs were then exposed to 10 nM thrombin for 6 hours (Figure 3.18C). The results show that transduction with dn-MEK1 significantly reduced thrombin-induced CCL2 production by 52±3.5% ($p<0.01$), whereas transduction with ca-MEK1 significantly increased the CCL2 basal production by 3.7±0.3 fold ($p<0.01$), but had no additive effect on thrombin-stimulated CCL2 production. These results provide further evidence that MEK1-ERK1/2 pathway is both necessary and sufficient for PAR_1-mediated CCL2 production in MLFs.

3.3.3 Effect of ERK1/2 on thrombin-induced CCL2 mRNA levels

To determine whether ERK1/2 mediates PAR_1-induced CCL2 production via the increase of CCL2 mRNA level, the effect of ERK1/2 inhibition by U0126 on thrombin-induced CCL2 mRNA levels was examined (Figure 3.19). MLFs were pre-incubated with U0126 (1 μm) for 30 minutes prior to stimulation with 10 nM thrombin for 2 hours. As shown in figure 3.19, at concentration of U0126 that blocked thrombin-induced ERK1/2 phosphorylation, thrombin-induced CCL2 mRNA accumulation was blocked by ~70%. The data suggest that ERK1/2 mediates thrombin-induced CCL2 production via a transcriptional mechanism.

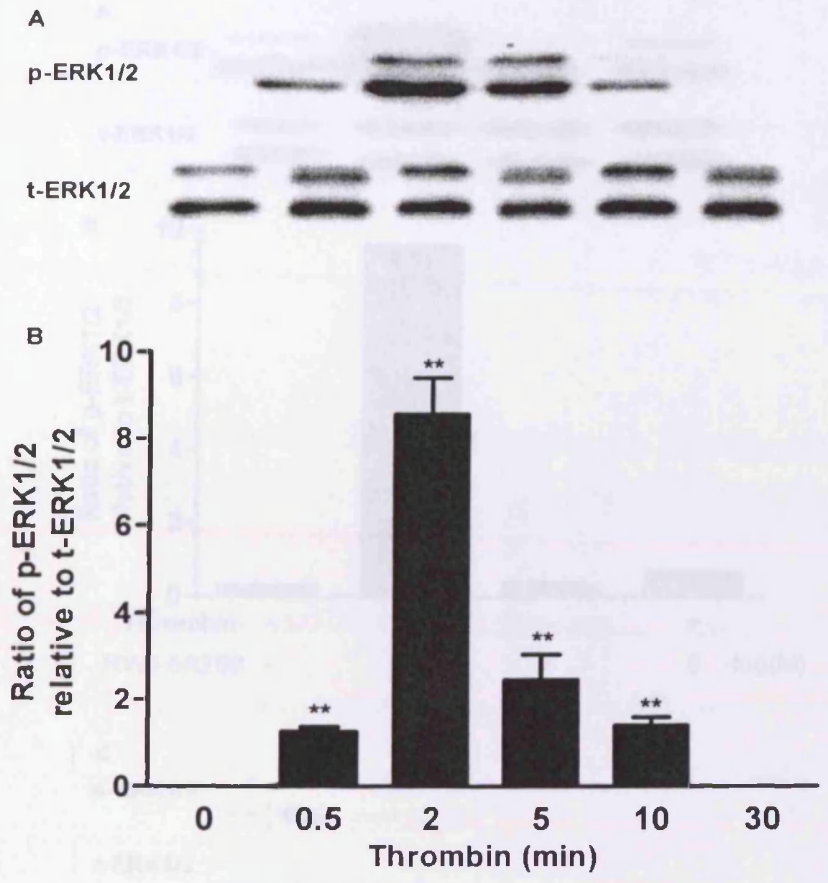


Figure 3.15 Time course of activation of extracellular mitogen-regulated kinases ERK1/2 by thrombin in MLFs.

Panel A shows time-course data for the effect of thrombin on MLF ERK1/2 phosphorylation. MLFs were exposed to thrombin (10 nM) for the indicated time periods. Phosphorylated ERK1/2 was assessed by western blotting using an anti phospho-ERK1/2 antibody (upper panel). Protein loading was verified by blotting with an anti-ERK1/2 antibody (lower panel). The relative increase in ERK1/2 phosphorylation normalized to total ERK1/2 was determined by performing quantitative densitometry (panel B). Data represent an average of three independent experiments ± SEM. ¨p<0.01, comparison with medium control at time zero.

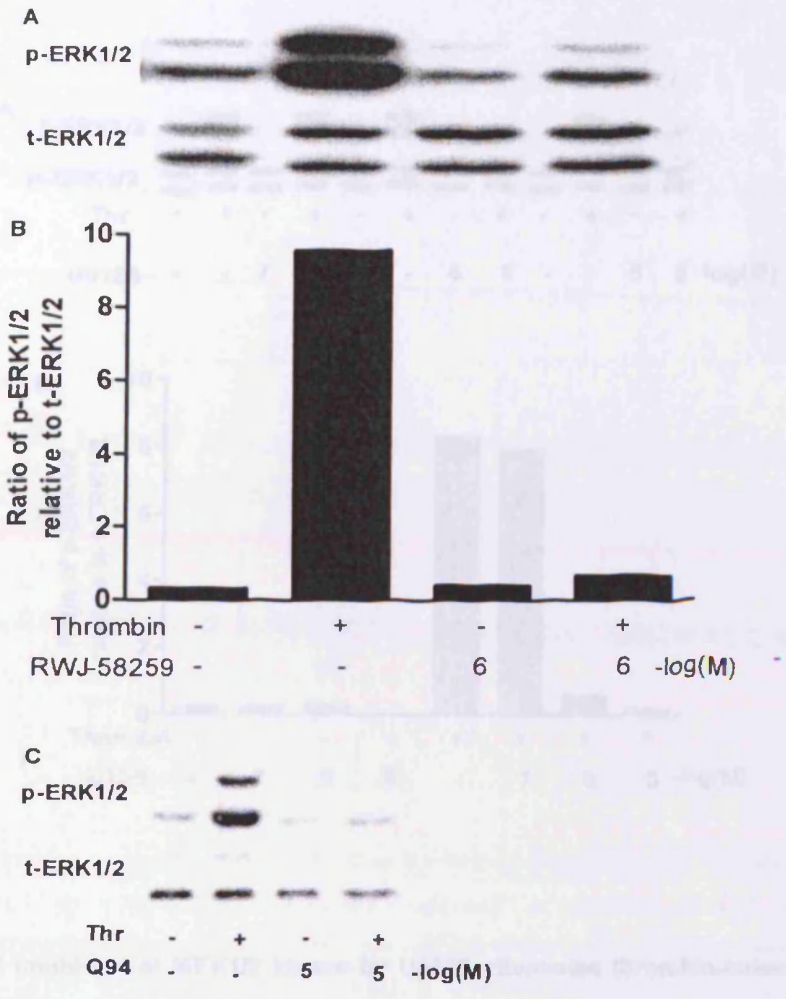


Figure 3.16 PAR_1 antagonism blocks thrombin-evoked ERK1/2 phosphorylation in MLFs. Panel A shows the effect of PAR_1 inhibition by RWJ-58259 on ERK1/2 phosphorylation in response to thrombin in MLFs. Cells were pre-incubated with RWJ-58259 (1 μM) for 15 min before exposure to 10 nM thrombin for 2 minutes. Phosphorylation of ERK1/2 was assessed by western blotting using an anti phospho-ERK1/2 antibody (upper panel). Protein loading was verified by blotting with an anti-ERK1/2 antibody (lower panel). The relative increase in ERK1/2 phosphorylation normalized to total ERK1/2 was determined by performing quantitative densitometry (panel B). Panel C shows the effect of Q94 on thrombin-induced ERK1/2 phosphorylation. Cells were pre-incubated with Q94 (10 μM) for 3 hours before exposure to 10 nM thrombin for 2 minutes. Phosphorylation of ERK1/2 was assessed by western blotting using an anti phospho-ERK1/2 antibody (upper panel). Protein loading was verified by blotting with an anti-ERK1/2 antibody (lower panel). Data show the representative of three experiments.

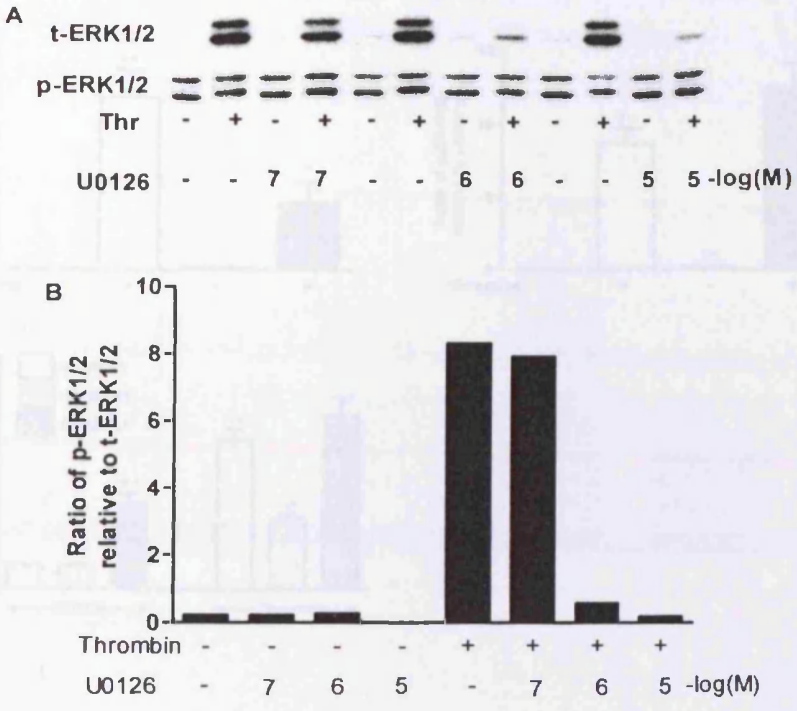


Figure 3.17 Inhibition of MEK1/2 kinase by U0126 attenuates thrombin-induced ERK1/2 phosphorylation.

Figure shows the effect of MEK1/2 kinase inhibition by U0126 on ERK1/2 phosphorylation induced by thrombin in MLFs. Cells were treated with increasing concentrations of U0126 for 30 minutes before stimulation with thrombin (10 nM) for 2 minutes. Phosphorylation of ERK1/2 was detected by using an anti phospho-ERK1/2 antibody (upper panel, A). Protein loading was verified by blotting with an anti-ERK1/2 antibody (lower panel, A). The relative increase of ERK1/2 phosphorylation normalized to total ERK1/2 was determined by performing quantitative densitometry (panel B). Data show the representative of three experiments.

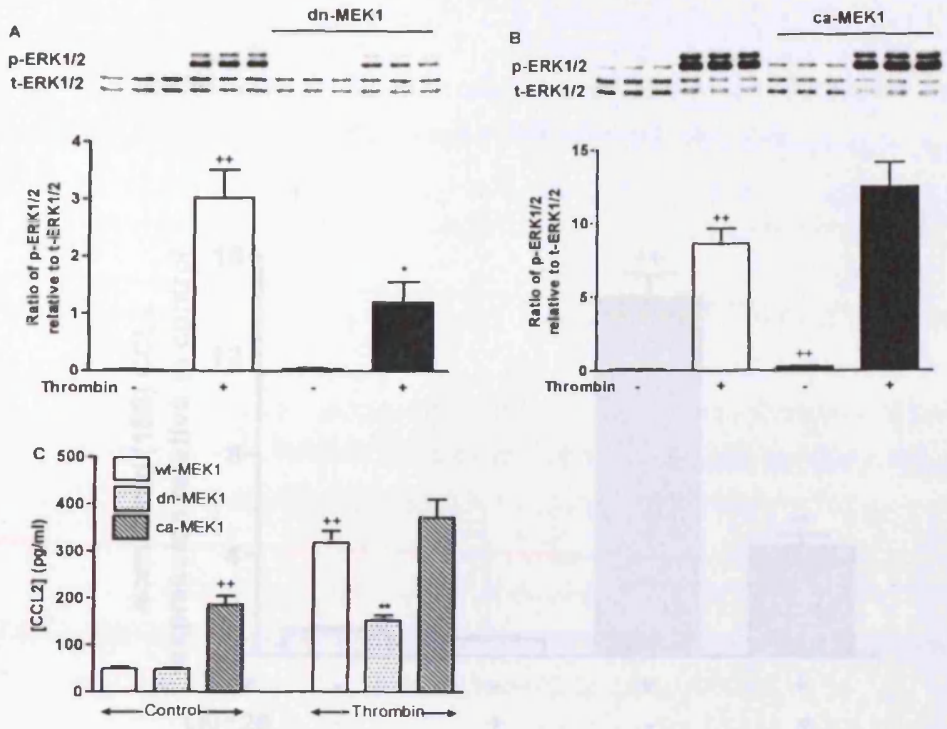


Figure 3.18 MEK1 is necessary and sufficient for thrombin-induced CCL2 production.
Panel A and B show the effects of wt-MEK1, dn-MEK1 or ca-MEK1 on thrombin-induced ERK1/2 phosphorylation. MLFs transduced with MEK1-pBabePuro constructs expressing the wt-MEK1, dn-MEK1 or ca-MEK1 were exposed to thrombin (10 nM) for 2 minutes. ERK1/2 phosphorylation was assessed by western blotting of cell lysates using an anti-phospho-ERK1/2 antibody (upper panel). Protein loading was verified by blotting with an anti-ERK1/2 antibody (lower panel). The graphs represent the densitometry analysis of the blots as a result of phospho-ERK1/2 relative to total ERK1/2 and are representative of three separate experiments performed with three replicates per sample. Panel C shows the effects of MEK1 constructs on thrombin-induced MLF CCL2 protein production. MLFs were transduced with MEK1-pBabePuro constructs expressing the wt-MEK1, dn-MEK1 or ca-MEK1. Cells were exposed to 10 nM thrombin for 6 hours. Levels of CCL2 protein production in supernatants were measured by ELISA. Data represent the mean ± SEM from triplicates, and show the representative of three independent experiments. $^{++}p<0.01$, comparison with basal CCL2 protein levels for the group with wt-MEK1. $^{**}p<0.01$, comparison with thrombin-induced CCL2 protein levels for the group with wt-MEK1.

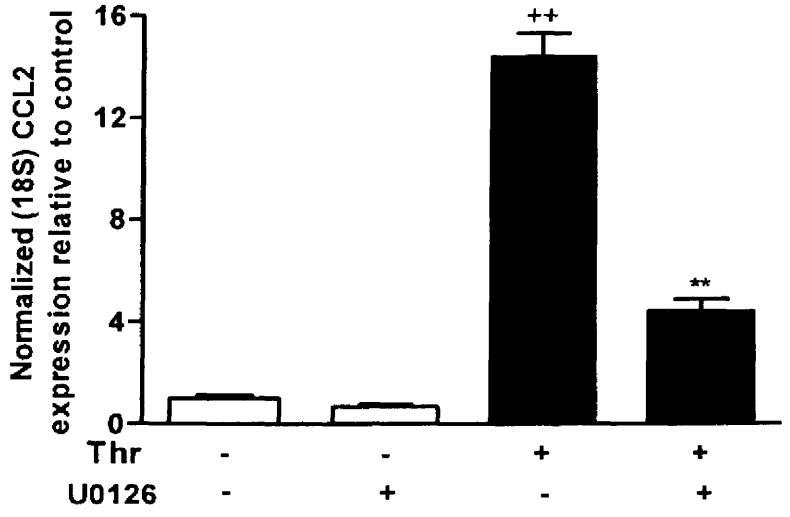


Figure 3.19 Inhibition of ERK1/2 inhiblted thrombin-induced CCL2 mRNA levels

Panel shows the effect of U0126 on thrombin-induced CCL2 mRNA levels. MLFs were exposed to thrombin (10 nM) for 2 hours with or without pre-incubation with U0126 (1μM), for 30 minutes. CCL2 mRNA levels were determined by qRT-PCR. Final concentrations of DMSO were kept constant for all treatment conditions (0.01% DMSO in DMEM). Data represent the mean ± SEM from triplicates, and show the representative of three independent experiments. $^{++}$ $p<0.01$, comparison with medium control; ** $p<0.01$, comparison with thrombin alone.

3.3.4 Summary

The results described in this section examining the role of MAP kinases in thrombin-induced MLF CCL2 protein release and mRNA levels showed that:

- Thrombin-induced CCL2 production is not dependent on p38 activation.
- JNK is also not involved in thrombin-induced CCL2 production.
- ERK1/2 pathway is necessary for mediating thrombin-induced CCL2 production as inhibition of ERK1/2 by both U0126 and PD98059 blocked PAR_1-induced CCL2 production in a concentration-dependent manner.
- The involvement of ERK1/2 in thrombin-induced CCL2 production is further confirmed by experiments showing that transduction of MLFs with dn-MEK1 significantly reduced thrombin-induced CCL2 production.
- ERK1/2 pathway is also sufficient for mediating thrombin-induced CCL2 production as transduction of MLFs with ca-MEK1 had no additive effect on thrombin-induced CCL2 production.
- ERK1/2 pathway is downstream of PAR_1 coupling to $G\alpha_q$ for mediating thrombin-induced CCL2 production.
- Inhibition of ERK1/2 inhibited thrombin-induced CCL2 mRNA levels.

In summary, ERK1/2 pathway downstream of PAR_1 coupling to $G\alpha_q$ is involved in thrombin-induced CCL2 production via a transcriptional mechanism.

3.4 The role of c-Raf and PKC in PAR_1-induced MLF CCL2 protein release and mRNA levels

It has been shown that PAR_1-$G\alpha_q$ interaction leads to the activation of ERK1/2 via the activation of both c-Raf and PKC in MLFs (Trejo *et al.*, 1996). In addition, PKC is a well-known downstream effector of $G\alpha_q$. I therefore performed experiments to examine the role of c-Raf and PKC in thrombin-induced MLF CCL2 protein release and mRNA levels.

3.4.1 Effect of c-Raf inhibition on PAR_1-induced CCL2 production

Cells were pre-incubated with c-Raf inhibitor for 30 minutes prior to stimulation with thrombin for 6 hours. The results show that c-Raf inhibitor inhibited thrombin-induced CCL2 release in a concentration-dependent manner from 1 μM (-Log [1 μM]=6) onwards (Figure 3.20A). At 10 μM (-Log [10 μM]=5), c-Raf inhibitor produced a 95 ± 1.2% reduction in thrombin induced-CCL2 production ($p<0.01$). The IC_{50} of c-Raf inhibitor was determined to be 1.5 μM for this response. To further determine whether c-Raf mediates thrombin-induced CCL2 production via ERK1/2 activation, the effect of c-Raf inhibition on ERK1/2 phosphorylation induced by thrombin was examined. Cells were pre-incubated with increasing concentrations of c-Raf inhibitor before exposure to thrombin for 2 minutes. As shown in Figure 3.20B, c-Raf inhibitor concentration-dependently inhibited thrombin-induced ERK1/2 phosphorylation with complete inhibition obtained at 3 μM. These data provide strong evidence that c-Raf mediates thrombin-induced CCL2 protein production via the activation of ERK1/2.

3.4.2 Effect of PKC inhibition on PAR_1-induced CCL2 production

In order to establish whether PAR_1-mediated CCL2 production is PKC-dependent, the effects of broad spectrum PKC inhibitors, Ro-318425 and GF109203X, on this response were examined. MLFs were pre-incubated with GF109203X or Ro-318425 for 30 minutes prior to stimulation with thrombin for 6 hours. The results show that both GF109203X (Figure 3.21 A) and Ro-318425 (Figure 3.21 B) blocked thrombin-induced CCL2 release in a concentration-dependent manner from 0.1 μM (-Log [0.3 μM]=7) onwards for GF109203X, and from 0.3 μM (-Log [0.3 μM]=6.5) onwards for Ro-318425. At 10 μM (-Log [10 μM]=5), GF109203X produced a complete reduction in thrombin induced-CCL2 production, whereas Ro-318425 produced a 82 ± 0.39% reduction ($p<0.01$) in this response. The IC_{50} of GF109203 and Ro-318425 were determined to be 0.73 μM and 0.93 μM, respectively, for this response. These data suggest that PAR_1-mediated CCL2 production is indeed PKC-dependent.

Previous studies performed with MLFs have shown that PAR_1 coupling to Ga_q can activate PKC and downstream c-Raf and ERK1/2 (Trejo, 1996), I next performed experiments to determine whether PKC mediates thrombin-induced CCL2 production via the activation of both c-Raf and ERK1/2. The effects of PKC inhibition on c-Raf and ERK1/2 phosphorylation induced by thrombin were examined by western blotting. Cells were pre-incubated with Ro-318425 for 30 minutes before exposure to thrombin for 2 minutes (for ERK1/2 phosphorylation) or 10 minutes (for c-Raf phosphorylation). As shown in Figure 3.22 A&B, Ro-318425 concentration-dependently inhibited ERK1/2 phosphorylation, and at 1 μM, Ro-318425 almost completely blocked thrombin-induced ERK1/2 phosphorylation (about 90%). Inhibition of PKC by Ro-318425 (1 μM) also completely inhibited c-Raf phosphorylation induced by thrombin (Figure 3.22 C).

These data provide strong evidence that PKC mediates thrombin-induced CCL2 protein production via the activation of c-Raf and ERK1/2. ERK1/2, c-Raf and PKC are in a linear pathway downstream of PAR_1 coupling to Ga_q in mediating thrombin-induced CCL2 protein production.

3.4.3 Effect of c-Raf and PKC inhibition on PAR_1-mediated CCL2 mRNA levels

To determine whether c-Raf and PKC are also involved in thrombin-induced CCL2 mRNA levels, I next examined the effects of c-Raf and PKC inhibition on this response (Figure 3.23). MLFs were pre-incubated with c-Raf inhibitor (3 μM, panel A) or Ro-318425 (1 μM, panel B) for 30 minutes prior to exposure to thrombin for 2 hours. The mRNA levels were assessed by qRT-PCR. As shown in Figure 3.23, at concentrations of c-Raf inhibitor and Ro-318425 that blocked thrombin-induced ERK1/2 phosphorylation, thrombin-induced CCL2 mRNA accumulation was blocked by 75% and 81% respectively. These data suggest that c-Raf and PKC are involved in thrombin-induced MLF CCL2 production via a transcriptional mechanism.

Taken together, data obtained so far demonstrate that PKC, c-Raf and ERK1/2 are in a linear pathway for thrombin-induced CCL2 gene expression and protein production.

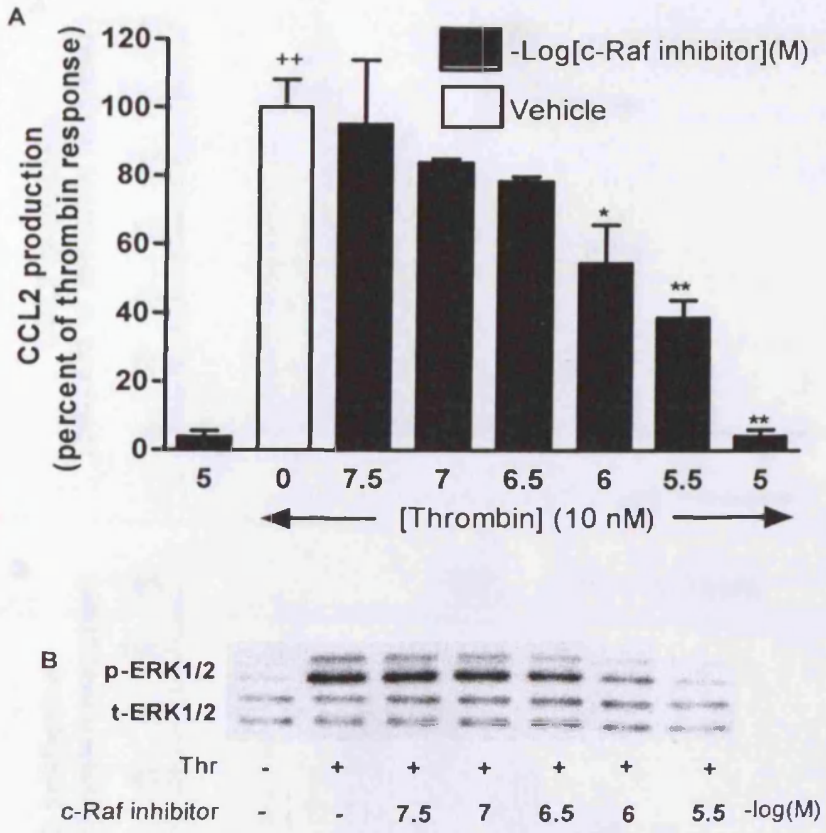


Figure 3.20 Inhibition of c-Raf by c-Raf kinase inhibitor inhibits thrombin-induced MLF CCL2 production and ERK1/2 phosphorylation.

Panel A shows the effect of c-Raf kinase inhibitor on CCL2 protein production in response to 10 nM thrombin, presented as a percentage of the maximal response obtained for thrombin with drug vehicle alone (0.1% DMSO in DMEM). Cells were treated with increasing concentrations of c-Raf kinase inhibitor for 30 minutes before exposure to thrombin for 6 hours. Final concentrations of DMSO were kept constant for all treatment conditions. The first bar represents the highest concentration of c-Raf kinase inhibitor and shows that this compound has no effect on basal CCL2 production. Negative log of the concentrations of c-Raf kinase inhibitor are presented. Data represent the mean ± SEM of triplicates. Panel B shows the effect of c-Raf kinase inhibitor on thrombin-induced ERK1/2 phosphorylation in MLFs. ERK1/2 phosphorylation was assessed by western blotting of cell lysates using an anti-phospho-ERK1/2 antibody (upper panel). Protein loading was verified by blotting with an anti-ERK1/2 antibody (lower panel). Data show the representative of three independent experiments. $^{++}p<0.01$, comparison with unstimulated cells; $^{*}p<0.05$ and $^{**}p<0.01$, comparison with thrombin alone.

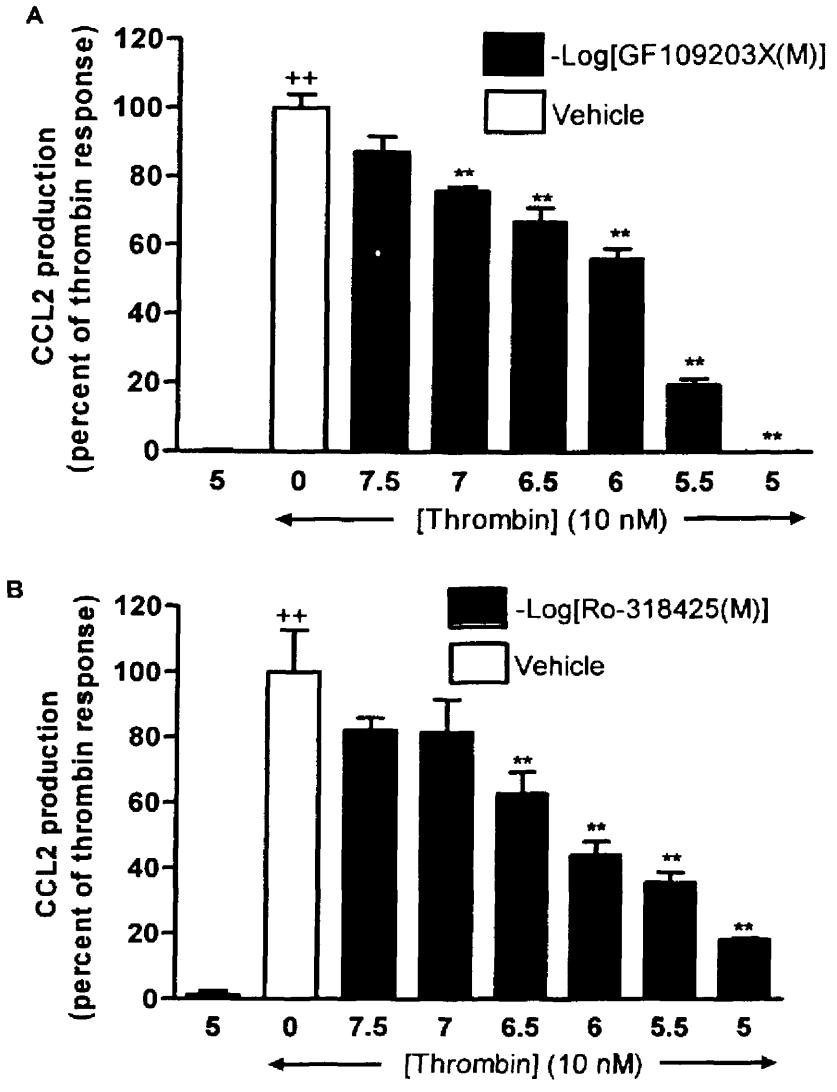


Figure 3.21 The protein kinase C (PKC) inhibition by GF109203X and Ro-318425 inhibits thrombin-induced CCL2 production.

Figure shows the effects of GF109203X and Ro-318425 on CCL2 protein production in response to thrombin. Data are presented as a percentage of the maximal response obtained for thrombin with drug vehicle alone (0.1% DMSO in DMEM). Cells were treated with increasing concentrations of inhibitors for 30 minutes before exposure to thrombin for 6 hours. Final concentrations of DMSO were kept constant for all treatment conditions. The first bars represent the highest concentration of inhibitors used and show that these compounds have no effect on basal CCL2 production. Negative log of the concentrations of inhibitors are presented. Data represent the mean ± SEM of triplicates, and show the representative of three independent experiments. $^{++}p<0.01$, comparison with unstimulated cells; $^{**}p<0.01$, comparison with thrombin alone.

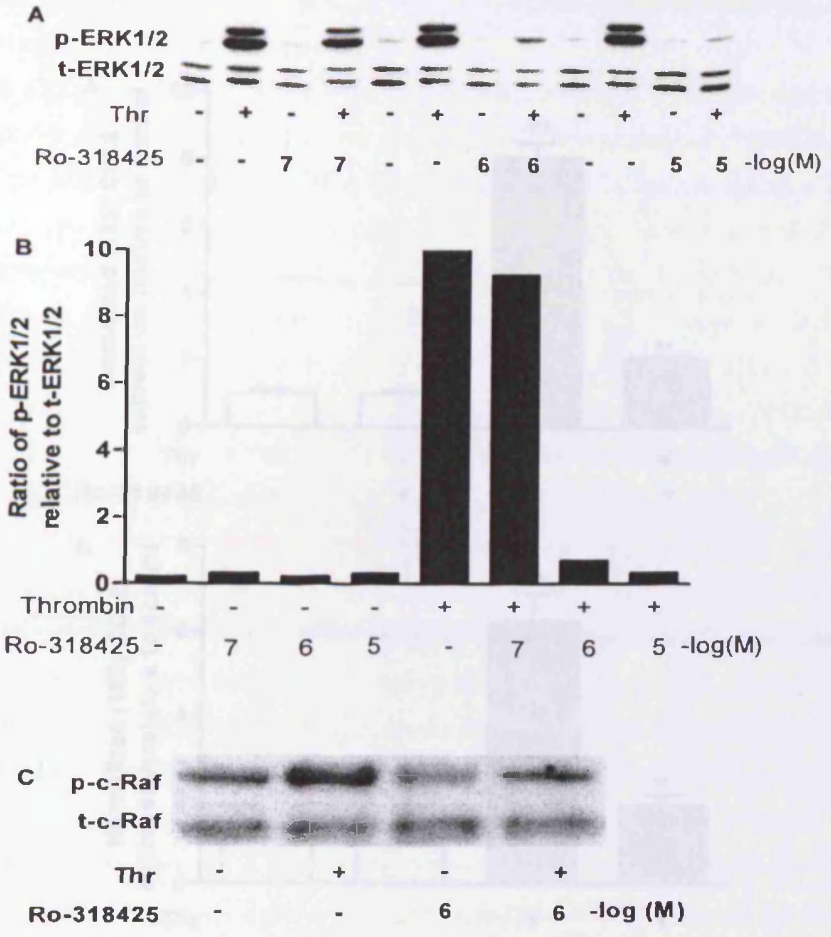


Figure 3.22 PKC inhibition by Ro-318425 attenuates thrombin-induced ERK1/2 and c-Raf phosphorylation.

Panel A shows the effect of Ro-318425 on thrombin-induced ERK1/2 phosphorylation. Cells were treated with increasing concentrations of Ro-318425 for 30 minutes before stimulation with thrombin (10 nM) for 2 minutes. ERK1/2 phosphorylation was assessed by western blotting of cell lysates using an anti-phospho-ERK1/2 antibody (upper panel). Protein loading was verified by blotting with an anti-ERK1/2 antibody (lower panel). The relative increase of ERK1/2 phosphorylation normalized to total ERK1/2 was determined by performing quantitative densitometry (Panel B). Panel C shows the effect of Ro-318425 on thrombin-induced MLF c-Raf phosphorylation. Cells were treated with Ro-318425 (1 μM) for 30 minutes before stimulation with thrombin (10 nM) for 10 minutes. c-Raf phosphorylation was assessed by western blotting of cell lysates using an anti-phospho-c-Raf antibody (upper panel). Protein loading was verified by blotting with an anti-c-Raf antibody (lower panel). The blot is representative of three separate experiments performed.

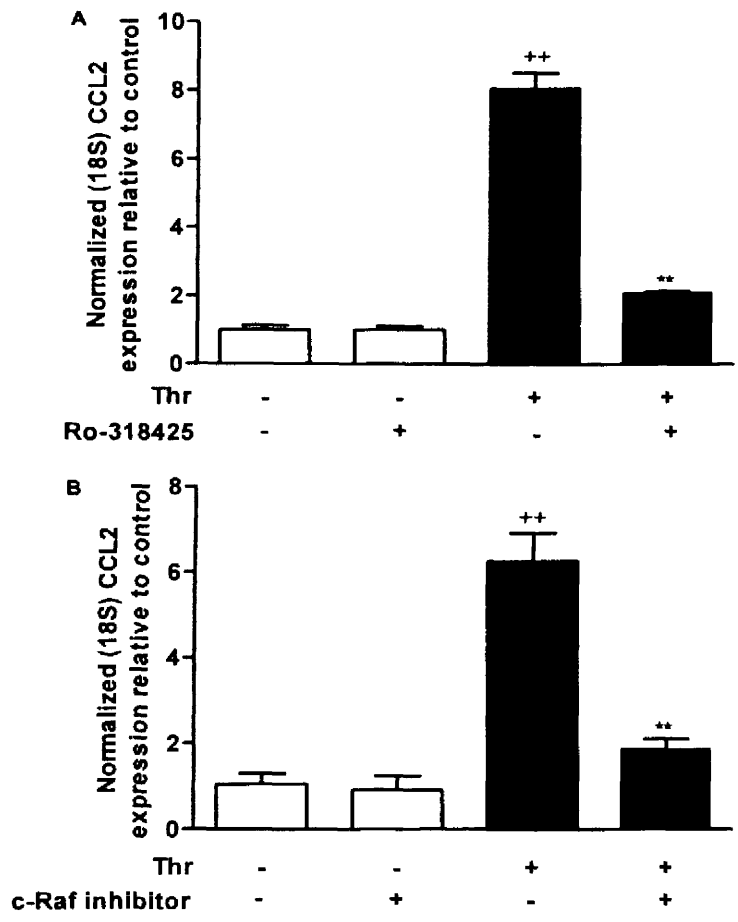


Figure 3.23 Inhibition of c-Raf and PKC inhibited thrombin-induced CCL2 mRNA levels
Panel A and B show the effects of Ro-318425 (A) and c-Raf inhibitor (B) on thrombin-induced CCL2 mRNA levels. MLFs were exposed to thrombin (10 nM) for 2 hours with or without pre-incubation with c-Raf inhibitor (3 μM) or Ro-318425 (1 μM) for 30 minutes. CCL2 mRNA levels were determined by qRT-PCR. Final concentrations of DMSO were kept constant for all treatment conditions (0.01% DMSO in DMEM). Data represent the mean ± SEM from triplicates, and show the representative of three independent experiments. $^{++}p<0.01$, comparison with medium control; $^{**}p<0.01$, comparison with thrombin alone.

3.4.4 Effect of Ca^{2+}-dependent PKC inhibitor Gö6976 on thrombin-induced CCL2 protein production and mRNA levels

In order to determine which PKC isoform (s) is/are involved in thrombin-induced CCL2 production, the effect of Gö6976 on this response was first examined. Gö6976 is widely accepted as a Ca^{2+}-dependent PKCs (PKC isoforms α, β1, β2, r) inhibitor (Martiny-Baron *et al.*, 1993). Cells were pre-incubated with increasing concentrations of Gö6976 (Figure 3.24) for 30 minutes before exposure to thrombin for 6 hours. The data obtained show that Gö6976 blocked PAR_1-mediated CCL2 protein production in a concentration-dependent manner from 0.1 μM onwards. At 10 μM, Gö6976 inhibited thrombin-induced CCL2 production by 83.4% ± 3.2% ($p<0.01$). The IC_{50} of Gö6976 was determined to be 0.5 μM for this response. These data suggest that Ca^{2+}-dependent PKC is involved in thrombin-induced CCL2 production.

In order to further confirm whether Ca^{2+}-dependent PKC mediates thrombin-induced CCL2 protein production via the transcriptional mechanism, the effect of Gö6976 on thrombin-induced CCL2 mRNA levels was examined (Figure 3.25, panel A). MLFs were pre-treated with Gö6976 for 30 minutes prior to exposure to thrombin (10 nM) for 2 hours. Surprisingly, inhibition of Ca^{2+}-dependent PKC did not inhibit thrombin-induced CCL2 mRNA levels, suggesting that Ca^{2+}-dependent PKC mediates thrombin-induced CCL2 protein production via a non-transcriptional mechanism. However, as shown in figure 3.21 B and figure 3.23 A, inhibition of general PKC isoforms by the broad spectrum inhibitors Ro-318425 inhibited thrombin-induced CCL2 protein release and CCL2 mRNA levels. These data suggest that Ca^{2+}-dependent PKC and Ca^{2+}-independent PKC mediate thrombin-induced CCL2 protein production via a different mechanism.

Previous results have shown that PKC mediates thrombin-induced CCL2 protein production via the activation of ERK1/2 (Figure 3.22 A). In order to determine whether Ca^{2+}-dependent PKC mediates thrombin-induced CCL2 release via ERK1/2, the effect of Gö6976 on thrombin-induced ERK1/2 phosphorylation was examined. As shown in Figure 3.25B, inhibition of Ca^{2+}-dependent PKC by Gö6976 did not inhibit ERK1/2 phosphorylation induced by thrombin, further suggesting that Ca^{2+}-dependent PKC and Ca^{2+}-independent PKC mediate thrombin-induced CCL2 protein production via a different mechanism.

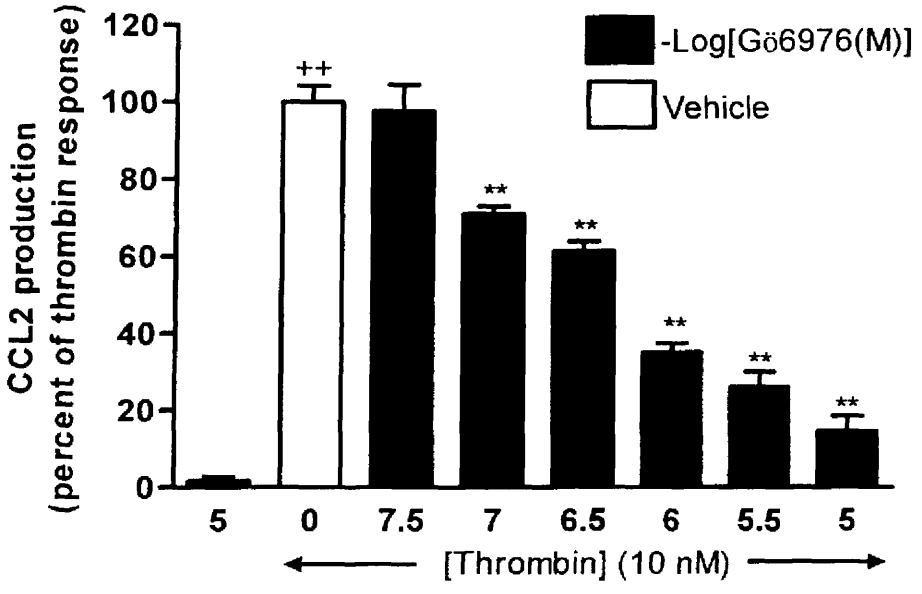
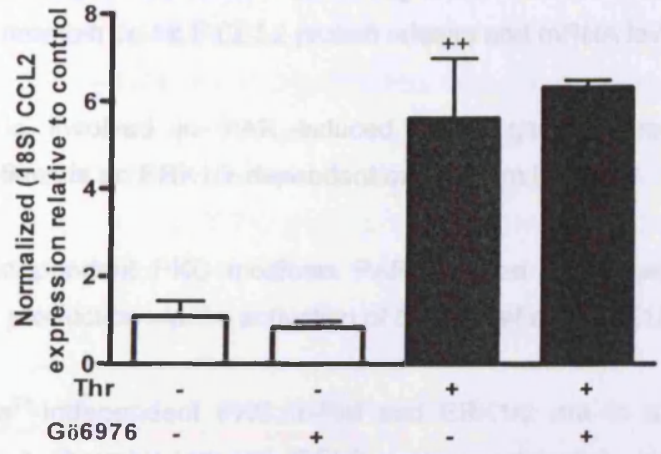


Figure 3.24 Inhibition of Ca^{2+}-dependent PKC by Gö6976 attenuates thrombin-induced CCL2 production.

Figure shows the effect of Gö6976 on thrombin-induced CCL2 protein production. Cells were pre-incubated with increasing concentrations of Gö6976 for 30 minutes before exposure to thrombin (10 nM) for 6 hours. Data are presented as a percentage of the maximal response obtained with thrombin and drug vehicle alone (0.1% DMSO in DMEM). Final concentrations of DMSO were kept constant for all treatment conditions. The first bar represents the highest concentration of Gö6976, and shows that this compound has no significant effect on basal CCL2 production. Negative log of the concentrations of Gö6976 are presented. Data represent the mean ± SEM from triplicates, and show the representative of three independent experiments. $^{++}p<0.01$, comparison with medium control; $^{**}p<0.01$, comparison with thrombin alone.

A



B

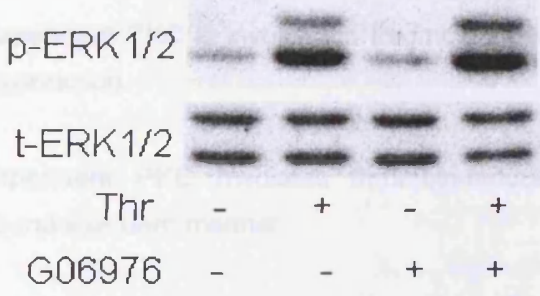


Figure 3.25 Inhibition of Ca^{2+}-dependent PKC does not interfere with thrombin-induced CCL2 mRNA levels and ERK1/2 phosphorylation.

Panel A shows the effect of Gö6976 on thrombin-induced CCL2 mRNA levels. MLFs were exposed to thrombin (10 nM) for 2 hours with or without pre-incubation with Gö6976 (1 μM) for 30 minutes. CCL2 mRNA levels were determined by qRT-PCR. Final concentrations of DMSO were kept constant for all treatment conditions (0.01% DMSO in DMEM). Data represent the mean ± SEM from triplicates, and show the representative of three independent experiments. Panel B shows the effect of Gö6976 on thrombin-induced ERK1/2 phosphorylation. Cells were treated with Gö6976 (1 μM) for 30 minutes before exposure to thrombin for 2 minutes. Phosphorylation of ERK1/2 was assessed by western blotting of cell lysates using an anti-phospho-ERK1/2 antibody (*upper panel*). Protein loading was verified by blotting with an anti-ERK1/2 antibody (*lower panel*). The blots are representative of three separate experiments performed. $^{++}p<0.01$, comparison with medium control.

3.4.5 Summary

The results described in this section examining the role of c-Raf and PKC in mediating the effects of thrombin on MLF CCL2 protein release and mRNA levels showed that:

- c-Raf is involved in PAR_1-induced CCL2 gene expression and protein production via an ERK1/2-dependent mechanism in MLFs.
- Ca^{2+}-independent PKC mediates PAR_1-induced CCL2 gene expression and protein production via the activation of both c-Raf and ERK1/2.
- The Ca^{2+}-independent PKC, c-Raf and ERK1/2 are in a linear pathway in mediating thrombin-induced CCL2 protein production via a transcriptional mechanism.
- Ca^{2+}-dependent PKC is involved in thrombin-induced CCL2 production but not gene expression.
- Ca^{2+}-dependent PKC mediates thrombin-induced CCL2 production in an ERK1/2-independent manner.

In summary, Ca^{2+}-independent PKC, c-Raf and ERK1/2 are in a linear pathway for thrombin-induced CCL2 gene expression and protein production; whereas Ca^{2+}-dependent PKC mediates thrombin-induced CCL2 production via a post-transcriptional mechanism.

3.5 The role of PLC-Ca^{2+} pathway and RhoA/Rho kinase in PAR_1-mediated protein release and mRNA levels

The data obtained so far point to a central role for $G\alpha_q$ and ERK1/2 in mediating the effects of PAR_1 activation on CCL2 production. The data obtained also suggest that Ca^{2+}-independent PKC and Ca^{2+}-dependent PKC mediate PAR_1-induced CCL2 release via different mechanisms. $G\alpha_q$ is well-known to trigger an increase in intracellular Ca^{2+} concentration by stimulating PLC-β activity, which eventually leads to the activation of Ca^{2+}-dependent PKC (Berridge, 1993). Moreover, thrombin is known to mediate Ca^{2+} signalling via PAR_1 in fibroblasts, and this can be inhibited by BAPTA (Trejo *et al.*, 1996; Tanaka *et al.*, 2004). It is therefore important to investigate the role of PLC-Ca^{2+} pathway in PAR_1-induced CCL2 production. In addition, although $G\alpha_{12/13}$ is generally considered as a major activator of RhoA/Rho kinase, there is emerging evidence that $G\alpha_q$ is also able to signal via Rho kinase by activating PLC-Ca^{2+} pathway (Singh *et a.*, 2007). Therefore, the roles of PLC-Ca^{2+} pathway and RhoA/Rho kinase in PAR_1-mediated CCL2 release were next examined.

3.5.1 Effect of PLC and Ca^{2+} inhibition on thrombin-induced CCL2 protein release and mRNA levels

To determine whether PLC and Ca^{2+} are also involved in thrombin-induced CCL2 production via a non-transcriptional mechanism, the same as Ca^{2+}-dependent PKC, the role of PLC and Ca^{2+} in thrombin-induced CCL2 production was first examined. Cells were treated with increasing concentrations of U73122 (PLC inhibitor, Figure 3.26A), or BAPTA-AM (Ca^{2+} chelator, Figure 3.26B) for 30 minutes before exposure to thrombin for 6 hours. As shown in Figure 3.26A, inhibition of PLC by U73122 inhibited thrombin-induced CCL2 production in a concentration-dependent manner from 1 μM, and a complete inhibition was obtained with U73122 at 6 μM. The IC_{50} of U73122 was determined to be 1.5 μM for this response. In order to verify the specificity of U73122, the effect of the inactive control compound for U73122, U73343, was also examined in parallel cultures, and was found to have no effect on this response at the highest concentrations used (Figure 3.26A). Similarly, the calcium chelator BAPTA-AM also blocked this response of thrombin in a concentration-dependent manner from 0.3 μM onwards (Figure 3.26B). At 3 μM, BAPTAM-AM attenuated this response by 72 ± 5% ($p<0.01$). The IC_{50} of BAPTA-AM was determined to be 1 μM for this response.

The role of PLC (Figure 3.27A) and Ca^{2+} (Figure 3.27B) in thrombin-induced CCL2 mRNA levels was next examined. Cells were pre-incubated with U73122 (2 μM) or BAPTA-AM (2 μM) for 30 minutes before exposure to thrombin for 2 hours. CCL2

mRNA levels were assessed by qRT-PCR. As shown in figure 3.27, inhibition of both PLC and Ca^{2+} by U73122 and BAPTA-AM had no effect on thrombin-induced CCL2 mRNA levels, suggesting that PLC and Ca^{2+} also mediate thrombin-induced CCL2 protein release via a non-transcriptional mechanism.

To further confirm that PLC and Ca^{2+} pathways mediate thrombin-induced CCL2 protein production via a different mechanism as indicated in Ca^{2+}-independent PKC pathway, the role of PLC (Figure 3.28A) and Ca^{2+} (Figure 3.28B) on thrombin-induced ERK1/2 phosphorylation was examined. Cells were pre-incubated with U73122 (2 μM) or BAPTA-AM (2 μM) for 30 minutes before exposure to thrombin for 2 minutes. ERK1/2 phosphorylation was measured by western blotting. As shown in figure 3.28, both inhibitors did not inhibit thrombin-induced ERK1/2 phosphorylation, suggesting that PLC and Ca^{2+} mediate thrombin-induced CCL2 protein release via an ERK1/2-independent mechanism.

Data obtained so far strongly suggest that the PLC, Ca^{2+} and Ca^{2+}-dependent PKC are involved in PAR_1-induced CCL2 release via a non-transcriptional mechanism and it is not via the activation of ERK1/2.

3.5.2 Effect of RhoA and Rho kinase inhibition on thrombin-induced CCL2 protein release and mRNA levels

As mentioned previously that $G\alpha_q$ is also able to signal via Rho kinase by activating the PLC-Ca^{2+} pathway (Singh *et al.*, 2007), the involvement of RhoA and Rho kinase in PAR_1-induced CCL2 release was next examined. Cells were treated with increasing concentrations of C3 exoenzyme (RhoA inhibitor, Figure 3.29) for 30 minutes before exposure to thrombin for 6 hours. The highest concentration of C3 exoenzyme used was 0.2 μg/ml as cells died at concentrations higher than 0.2 μg/ml. The data obtained show that C3 exoenzyme blocked PAR_1-mediated CCL2 production in a concentration-dependent manner from 0.02 μg/ml onwards. At 0.2 μg/ml, C3 exoenzyme significantly inhibited this response of thrombin by 43 ± 0.2% ($p<0.01$). The IC_{50} of C3 exoenzyme was determined to be 0.2 μg/ml for this response. As Rho kinase is a well-known downstream effector of RhoA, the involvement of Rho kinase in this response was next examined. Two structurally un-related Rho kinase inhibitors, Y-27632 and H-1152, were used. Inhibition of Rho kinase with these two inhibitors inhibited thrombin-induced CCL2 protein production in a concentration-dependent manner from 3 μM and 1 μM onwards, respectively. At 30 μM, Y-27632 blocked this response by 85.5 ± 7.07% and the IC_{50} of Y-27632 was determined to be 3 μM for this response (Figure 30A). H-1152

at 10 μM blocked this response by 86.4 ± 4.36% and the IC_{50} of H-1152 was determined to be 2.7 μM for this response (Figure 30B). In order to determine the mechanism in which RhoA/Rho kinase mediates thrombin-induced CCL2 production, the role of RhoA/Rho kinase in thrombin-induced CCL2 mRNA levels was next examined (Figure 3.31). Cells were pre-incubated with C3 exoenzyme (0.2 μg/ml, Figure 3.31A) or Y-27632 (3 μM, Figure 3.31B) for 30 minutes before exposure to thrombin for 2 hours. CCL2 mRNA levels were assessed by qRT-PCR. The results show that inhibition of RhoA and Rho kinase did not inhibit the increase of CCL2 mRNA levels induced by thrombin, suggesting that RhoA/Rho kinase mediates thrombin-induced CCL2 protein production via a post-transcriptional mechanism.

In order to determine whether RhoA and Rho kinase influence thrombin-induced CCL2 production via the activation of ERK1/2, the effects of RhoA (Figure 3.32A) and Rho kinase (Figure 3.32 B) inhibition on thrombin-induced ERK1/2 phosphorylation were examined. Cells were pre-incubated with C3 exoenzyme (0.2 μg/ml) or Y-27632 (3 μM) for 30 minutes before exposure to thrombin for 2 minutes. Figure 3.31 showed that none of these two inhibitors interfered with thrombin-induced ERK1/2 phosphorylation, indicating that RhoA and Rho kinase mediate thrombin-induced CCL2 production in an ERK1/2-independent manner.

Data obtained so far strongly suggest that RhoA/Rho kinase mediate thrombin-induced CCL2 production via a post-transcriptional mechanism and it is via an ERK1/2-independent manner.

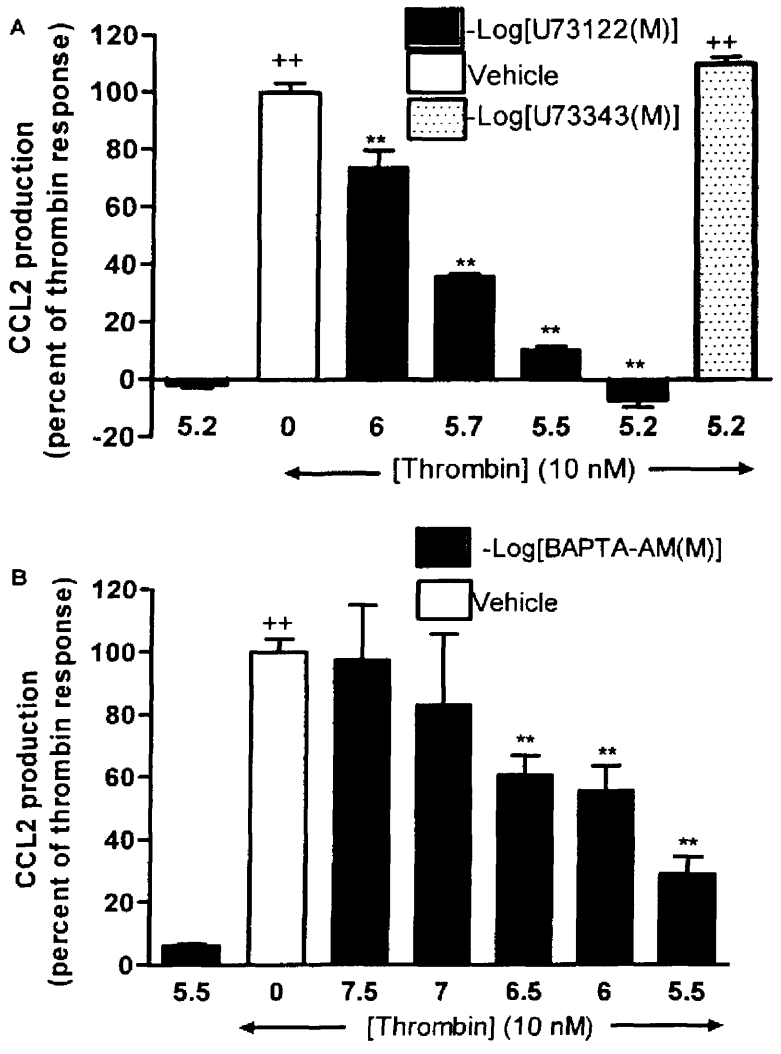


Figure 3.26 Inhibition of PLC (U73122) and Ca^{2+} (BAPTA1-AM) attenuates thrombin-induced CCL2 production.

Figure shows the effects of U73122, negative control U73343 and BAPTA-AM on thrombin-induced CCL2 protein release. Cells were pre-incubated with increasing concentrations of inhibitors for 30 minutes before exposure to thrombin (10 nM) for 6 hours. Data are presented as a percentage of the maximal response obtained with thrombin and drug vehicle alone (0.1% DMSO in DMEM). Final concentrations of DMSO were kept constant for all treatment conditions. The first bars represent the highest concentrations of inhibitors used, and show that these compounds have no significant effect on basal CCL2 production. Negative log of the concentrations of inhibitors are presented. Data represent the mean ± SEM from triplicates, and show the representative of three independent experiments. $^{++}p<0.01$, comparison with medium control; $^{**}p<0.01$, comparison with thrombin alone.

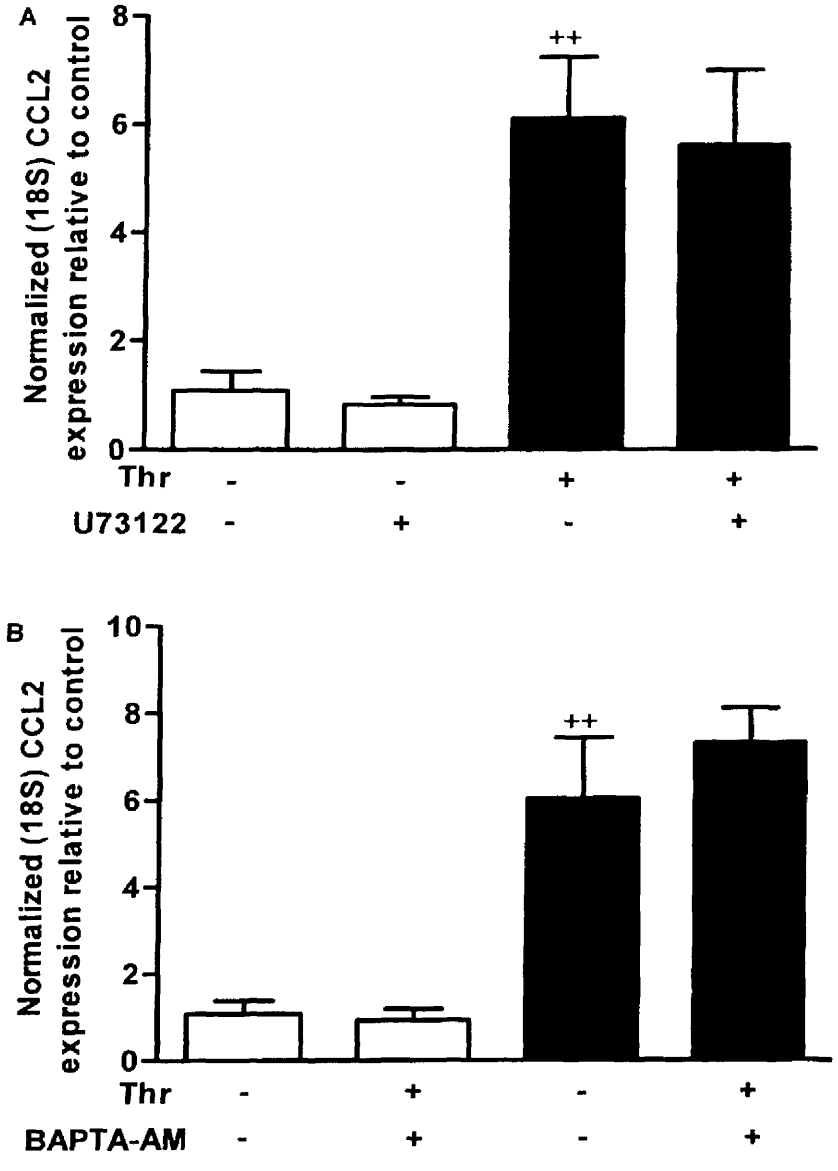


Figure 3.27 Inhibition of PLC (U73122) and Ca^{2+} (BAPTA-AM) does not interfere with thrombin-induced CCL2 mRNA levels.

Panel A and B show the effects of U73122 (A) and BAPTA-AM (B) on thrombin-induced CCL2 mRNA levels. MLFs were exposed to thrombin (10 nM) for 2 hours with or without pre-incubation with U73122 (2 μM) or BAPTA1-AM (2 μM) for 30 minutes. CCL2 mRNA levels were determined by qRT-PCR. Final concentrations of DMSO were kept constant for all treatment conditions (0.01% DMSO in DMEM). Data represent the mean ± SEM from triplicates, and show the representative of three independent experiments. $^{++}$ $p<0.01$, comparison with medium control.

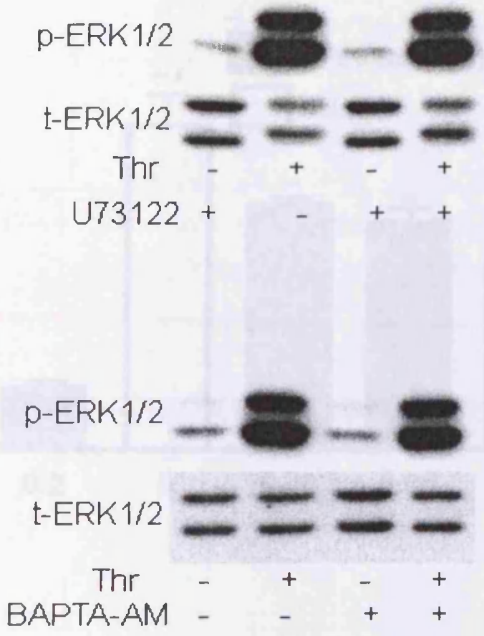


Figure 3.28 Inhibition of PLC (U73122) and Ca^{2+} (BAPTA-AM) does not interfere with thrombin-induced ERK1/2 phosphorylation.

Panel A and B show the effects of U73122 and BAPTA-AM on thrombin-induced ERK1/2 phosphorylation. Cells were treated with U73122 (2 μM) or BAPTA-AM (2 μM) for 30 minutes before exposure to thrombin for 2 minutes. Phosphorylation of ERK1/2 was assessed by western blotting of cell lysates using an anti-phospho-ERK1/2 antibody (*upper panel, A&B*). Protein loading was verified by blotting with an anti-ERK1/2 antibody (*lower panel, A&B*). The blots are representative of three separate experiments performed.

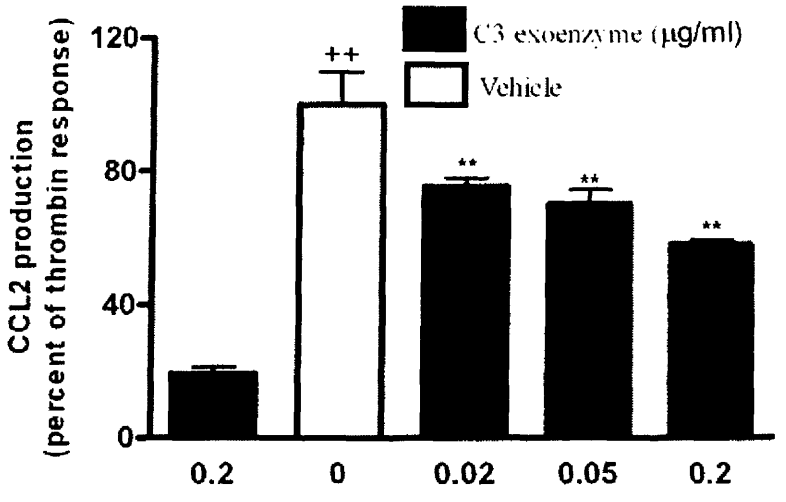


Figure 3.29 Inhibition of RhoA (C3 exoenzyme) attenuates thrombin-induced CCL2 production.

Figure shows the effect of C3 exoenzyme on thrombin-induced CCL2 protein release. Cells were pre-incubated with increasing concentrations of C3 exoenzyme for 30 minutes before exposure to thrombin (10 nM) for 6 hours. Data are presented as a percentage of the maximal response obtained with thrombin and drug vehicle alone. The first bar represents the highest concentration of C3 exoenzyme used, and shows that this compound has no significant effect on basal CCL2 production. Data represent the mean ± SEM from triplicates, and show the representative of three independent experiments. $^{++}p<0.01$, comparison with medium control; $^{**}p<0.01$, comparison with thrombin alone.

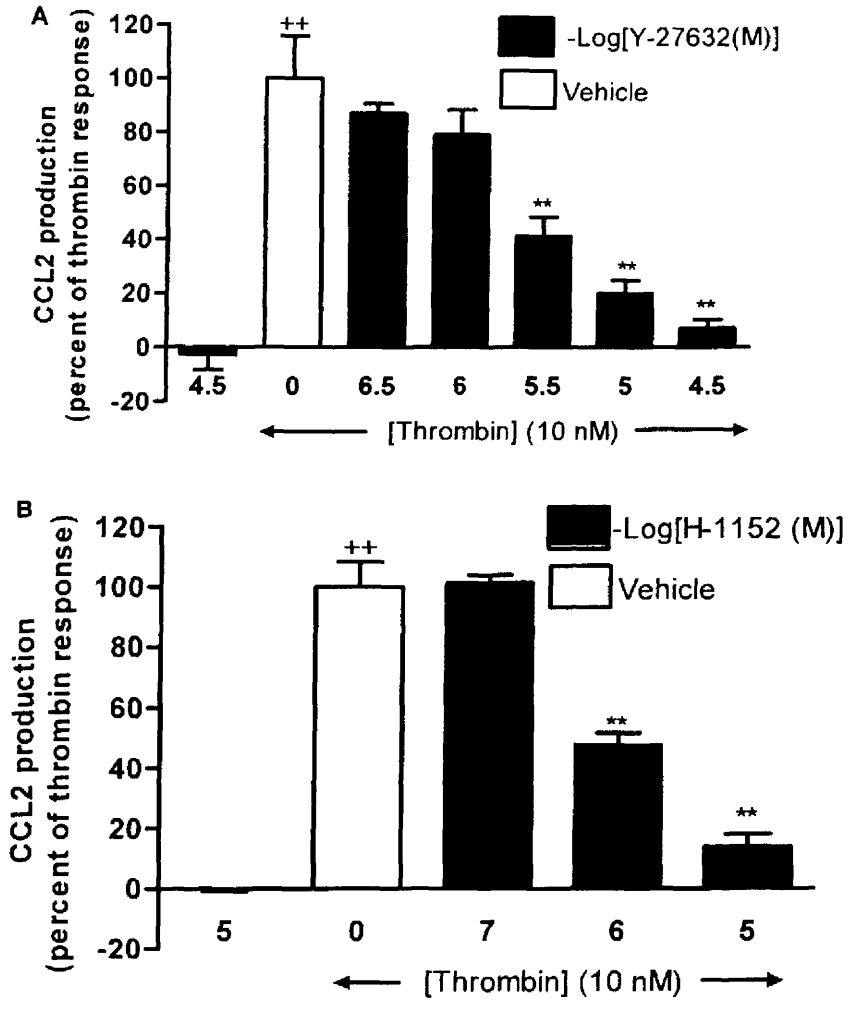


Figure 3.30 Inhibition of Rho kinase by Y-27632 and H-1152 attenuates thrombin-induced CCL2 production.

Panel A and B show the effects of Y-27632 and H-1152 on thrombin-induced CCL2 protein release. Cells were pre-incubated with increasing concentrations of inhibitors for 30 minutes before exposure to thrombin (10 nM) for 6 hours. Data are presented as a percentage of the maximal response obtained with thrombin and drug vehicle alone (1% DMSO in DMEM for Y-27632, H-1152 is dissolved in H_20). Final concentrations of DMSO were kept constant for all treatment conditions. The first bars represent the highest concentration of inhibitors used, and show that these compounds have no significant effects on basal CCL2 production. Negative log of the concentrations of inhibitors are presented. Data represent the mean ± SEM from triplicates, and show the representative of three independent experiments. $^{++}p<0.01$, comparison with medium control; $^{**}p<0.01$, comparison with thrombin alone.

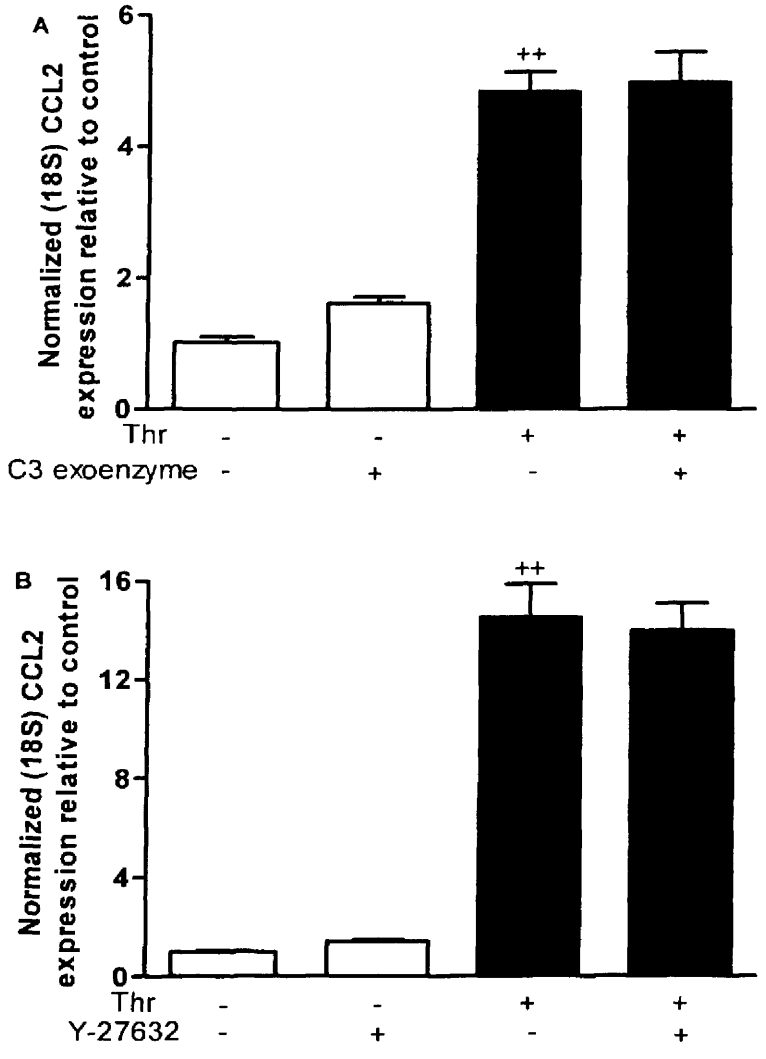


Figure 3.31 Inhibition of RhoA (C3 exoenzyme) and Rho kinase (Y-27632) does not interfere with thrombin-induced CCL2 mRNA levels.

Panel A and B show the effects of C3 exoenzyme (A) and Y-27632 (B) on thrombin-induced CCL2 mRNA levels. Cells were treated with C3 exoenzyme (0.2 μg/ml) or Y-27632 (3 μM) for 30 min before exposure to thrombin for 2 hours. CCL2 mRNA levels were determined by qRT-PCR. Data represent the mean ± SEM from triplicates, and show the representative of three independent experiments. $^{++}p<0.01$, comparison with medium control.

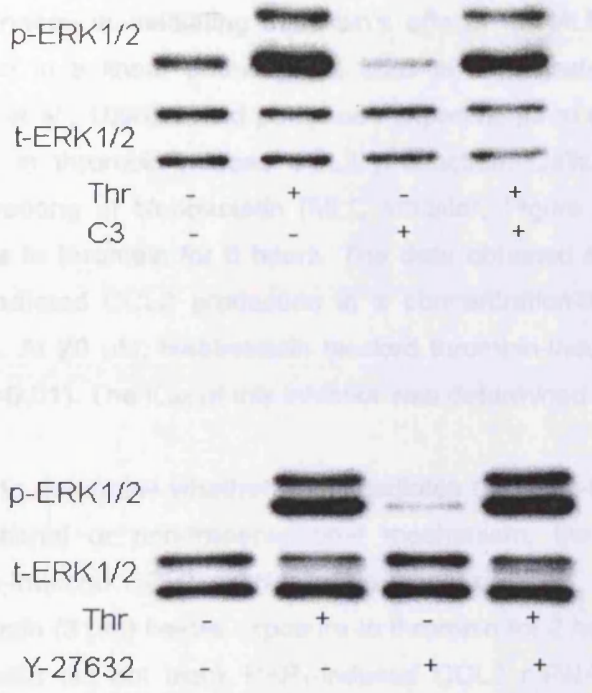


Figure 3.32 Inhibition of RhoA (C3 exoenzyme) and Rho kinase (Y-27632) does not interfere with thrombin-induced ERK1/2 phosphorylation.

Panel A and B show the effects of C3 exoenzyme (A) and Y-27632 (B) on thrombin-induced ERK1/2 phosphorylation. Cells were treated with C3 exoenzyme (0.2 µg/ml) or Y-27632 (3 µM) for 30 minutes before exposure to thrombin for 2 minutes. Phosphorylation of ERK1/2 was assessed by western blotting of cell lysates using an anti-phospho-ERK1/2 antibody (*upper panel, A&B*). Protein loading was verified by blotting with an anti-ERK1/2 antibody (*lower panel*, A&B). The blots are representative of three separate experiments performed.

3.5.3 Effect of MLC inhibition on thrombin-induced CCL2 protein release and mRNA levels

Data obtained so far have strongly suggested that PLC, Ca^{2+}, Ca^{2+}-dependent PKC, RhoA and Rho kinase mediate thrombin-induced CCL2 production in an ERK1/2-independent manner and via a non-transcriptional mechanism. Similarities among those kinases in mediating thrombin's effects on MLFs suggest that these kinases might act in a linear pathway. As MLC is a downstream substrate of Rho kinase (Amano *et al.*, 1996), I next performed experiments to determine whether MLC is also involved in thrombin-induced CCL2 production. Cells were treated with increasing concentrations of blebbestatin (MLC inhibitor, Figure 3.33A) for 30 minutes before exposure to thrombin for 6 hours. The data obtained show that this inhibitor blocked PAR_1-mediated CCL2 production in a concentration-dependent manner from 1 μM onwards. At 20 μM, blebbestatin blocked thrombin-induced CCL2 production by 72 ± 4.7% ($p<0.01$). The IC_{50} of this inhibitor was determined to be 4.9 μM for this response.

In order to determine whether MLC mediates thrombin-induced CCL2 production via a transcriptional or non-transcriptional mechanism, the effect of MLC inhibition on thrombin-induced CCL2 mRNA levels was examined. Cells were pre-incubated with blebbestatin (3 μM) before exposure to thrombin for 2 hours. As shown in figure 3.32B, blebbestatin did not block PAR_1-induced CCL2 mRNA levels, suggesting that MLC mediates thrombin-induced CCL2 production also via a post-transcriptional mechanism.

3.5.4 PLC-Rho kinase pathway mediates thrombin-induced CCL2 production via the activation of MLC

To determine whether PLC, Ca^{2+}, Ca^{2+}-dependent PKC, RhoA and Rho kinase are in a linear pathway in mediating thrombin's effects on MLFs via the activation of MLC, the effect of PLC, Ca^{2+}, Ca^{2+}-dependent PKC, RhoA and Rho kinase inhibition on thrombin-induced MLC phosphorylation was examined. Figure 3.34A shows that thrombin induced MLC phosphorylation within 10 minutes of stimulation and that this response was abolished by U73122, BAPTA-AM, Gö679 and Y-27632 (Figure 3.34B). These data indicate that PLC, Ca^{2+}, Ca^{2+}-dependent PKC, RhoA signal to Rho kinase rather than ERK1/2 in mediating the effects of thrombin on CCL2 release. To further confirm that PLC-Rho kinase pathway is downstream of PAR_1-coupling to $G\alpha_q$, the effect of $G\alpha_q$ selective PAR_1 antagonist Q94 on thrombin-induced MLC phosphorylation was next examined (Figure 3.34C). Data show that Q94 completely abolished thrombin-induced MLC phosphorylation, suggesting that the PLC-Rho kinase pathway

acts downstream of PAR_1 coupling to $G\alpha_q$ in mediating thrombin-induced CCL2 production.

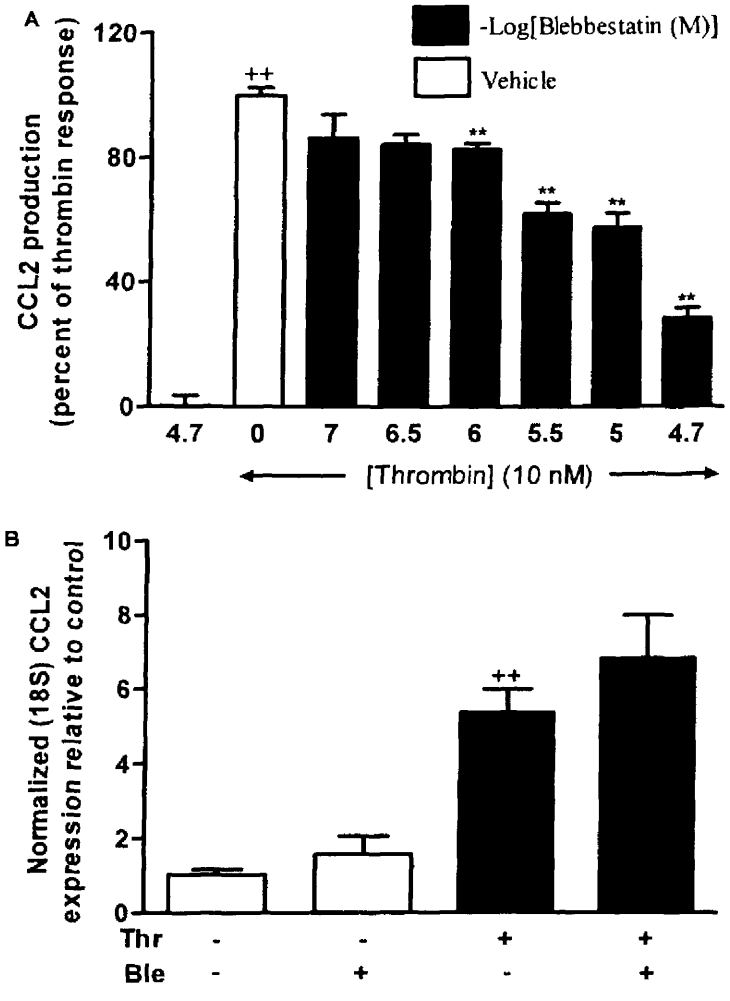


Figure 3.33 Inhibition of MLC by blebbestatin attenuates thrombin-induced CCL2 production, but not CCL2 mRNA levels.

Panel A shows the effect of blebbestatin on thrombin-induced CCL2 protein release. Cells were pre-incubated with increasing concentrations of blebbestatin for 30 minutes before exposure to thrombin (10 nM) for 6 hours. Data are presented as a percentage of the maximal response obtained with thrombin and drug vehicle alone (0.5% DMSO in DMEM). Final concentrations of DMSO were kept constant for all treatment conditions. The first bar represents the highest concentration of blebbestatin, and shows that this compound has no significant effect on basal CCL2 production. Negative log of the concentrations of blebbestatin are presented. Panel B shows the effect of blebbestatin on thrombin-induced CCL2 mRNA levels. MLFs were exposed to thrombin (10 nM) for 2 hours with or without pre-incubation with blebbestatin (3 μM) for 30 minutes. CCL2 mRNA levels were determined by qRT-PCR. Data represent the mean ± SEM

from triplicates, and show the representative of three independent experiments. $^{++}p<0.01$, comparison with medium control; $^{--}p<0.01$, comparison with thrombin alone.

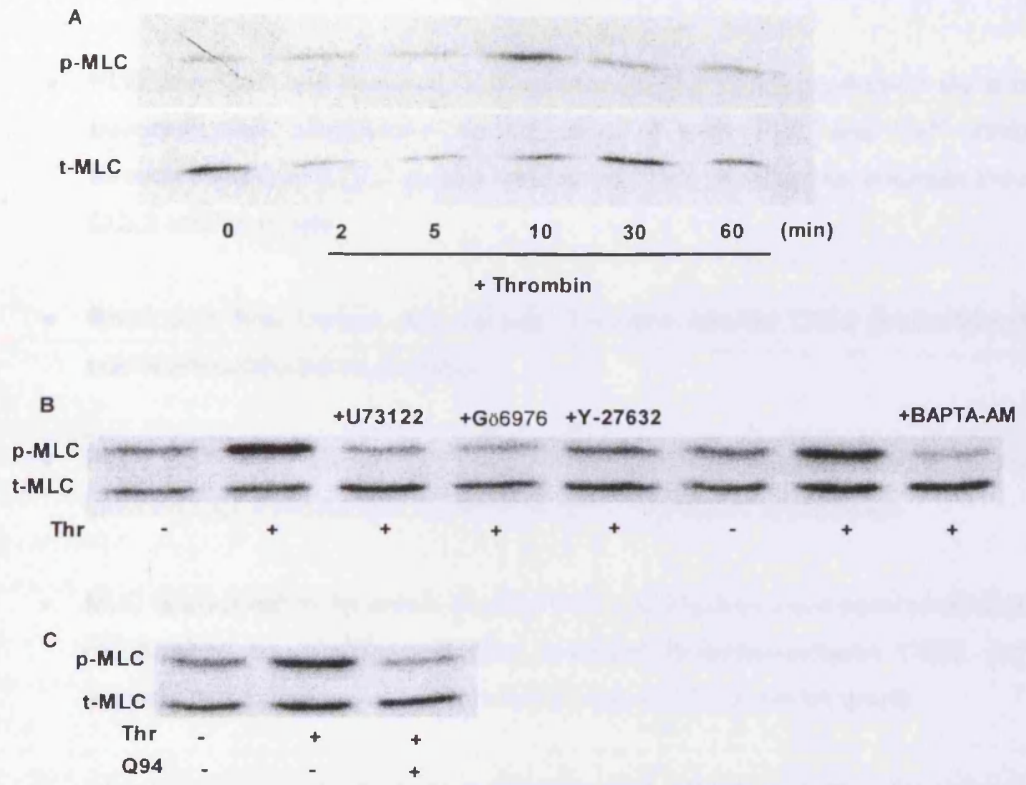


Figure 3.34 Thrombin induces MLC phosphorylation in a time-dependent manner and it is inhibited by the inhibition of PLC, Ca^{2+}, Ca^{2+}-dependent PKC, Rho kinase and the inhibition of PAR_1 coupling to $G\alpha_q$ by Q94 .

Panel A shows the effect of thrombin on MLC phosphorylation in MLFs. MLFs were exposed to thrombin (10 nM) for the indicated time periods. Panel B and C show the effects of U73122 (2 μM), BAPTA-AM (2 μM), Gö6976 (1 μM), Y-27632 (3 μM) and Q94 (10 μM) on thrombin-induced MLC phosphorylation in MLFs. Cells were pre-incubated with these inhibitors for 30 minutes followed by thrombin stimulation for 10 minutes. MLC phosphorylation was assessed by western blotting using an anti phosphor-MLC antibody (upper panel). Protein loading control was verified by blotting with a total MLC antibody (lower panel). Representative blots of three separate experiments are presented.

3.5.5 Summary

The results described in this section examining the role of PLC-Ca^{2+} pathway and RhoA/Rho kinase in thrombin-induced MLF CCL2 protein release and mRNA levels showed that:

- PLC and Ca^{2+} are involved in thrombin-induced CCL2 production via a post-transcriptional mechanism as inhibition of both PLC and Ca^{2+} inhibited thrombin-induced CCL2 protein release, but had no effect on thrombin-induced CCL2 mRNA levels.

- RhoA and Rho kinase also mediate thrombin-induced CCL2 production via a post-transcriptional mechanism.

- PLC, Ca^{2+}, Ca^{2+}-dependent PKC, RhoA and Rho kinase mediate thrombin-induced CCL2 production via an ERK1/2-independent mechanism.

- MLC is involved in thrombin-induced CCL2 production via a post-transcriptional mechanism as inhibition of MLC inhibited thrombin-induced CCL2 protein release, but had no effect on thrombin-induced CCL2 mRNA levels.

- Inhibition of PLC, Ca^{2+}, Ca^{2+}-dependent PKC, RhoA and Rho kinase inhibited thrombin-induced MLC phosphorylation, suggesting that these kinases are in a linear pathway for mediating thrombin-induced CCL2 production via a MLC-dependent mechanism.

- Inhibition of PAR_1 coupling to $G\alpha_q$ by the novel $G\alpha_q$-selective PAR_1 antagonist Q94 inhibited thrombin-induced MLC phosphorylation, suggesting that MLC pathway is downstream of PAR_1 coupling to $G\alpha_q$ for mediating thrombin-induced CCL2 production in MLFs.

In summary, PLC-Rho kinase pathway mediates thrombin-induced CCL2 protein production via a post-transcriptional mechanism, possibly via protein secretion. This pathway is also downstream of PAR_1 coupling to $G\alpha_q$.

3.6 Inhibition of Rho kinase by Y-27632 has no effect on intracellular protein production

The results obtained thus far demonstrate an essential role for ERK1/2 in PAR_1 activation-induced CCL2 production by influencing CCL2 mRNA levels; whereas the Rho kinase pathway acts via a post-transcriptional mechanism. To begin to elucidate the mechanism by which the Rho kinase pathway influences this response, immunocytofluoresence was employed to examine thrombin-induced intracellular CCL2 protein production.

MLFs seeded in the 8-well tissue culture chambers were pre-incubated with U0126 (10 μM) or Y-27632 (10 μM) for 30 minutes before exposure to thrombin for 3 hours. The chamber was then fixed and plotted with an anti-CCL2 antibody and normal goat IgG as isotype control. Figure 3.35 shows that unstimulated cells yield no immunostaining or a very weak signal for CCL2 (Figure 3.35B). In contrast, following stimulation with thrombin for 3 hours, up to 60% of cells were brightly stained as would be expected for cells which are actively synthesizing CCL2 (Figure 3.35C). The effect of thrombin was almost completely blocked in the presence of U0126 with only 5-10% of cells displaying a very weak signal for CCL2 (Figure 3.35D). In contrast, in the presence of the Rho kinase inhibitor, Y-27632, up to 60% of cells stained with CCL2 antibody brightly (Figure 3.35E), indicating that these cells are actively synthesizing CCL2. The isotype control was completely negative indicating that the signal obtained with the CCL2 antibody was specific (Figure 3.35A). The mean pixel intensity of three randomly chosen fields of view was calculated for each group (Figure 3.35F), and the data are presented as fold change over control. Thrombin induced a 2.8 ± 0.1 fold increase in fluorescent signal over control and this was significantly inhibited by U0126. In contrast, pre-treatment of cells with Y-27632 did not block thrombin-induced intracellular CCL2 accumulation (3.2 ± 0.5 fold increase over control).

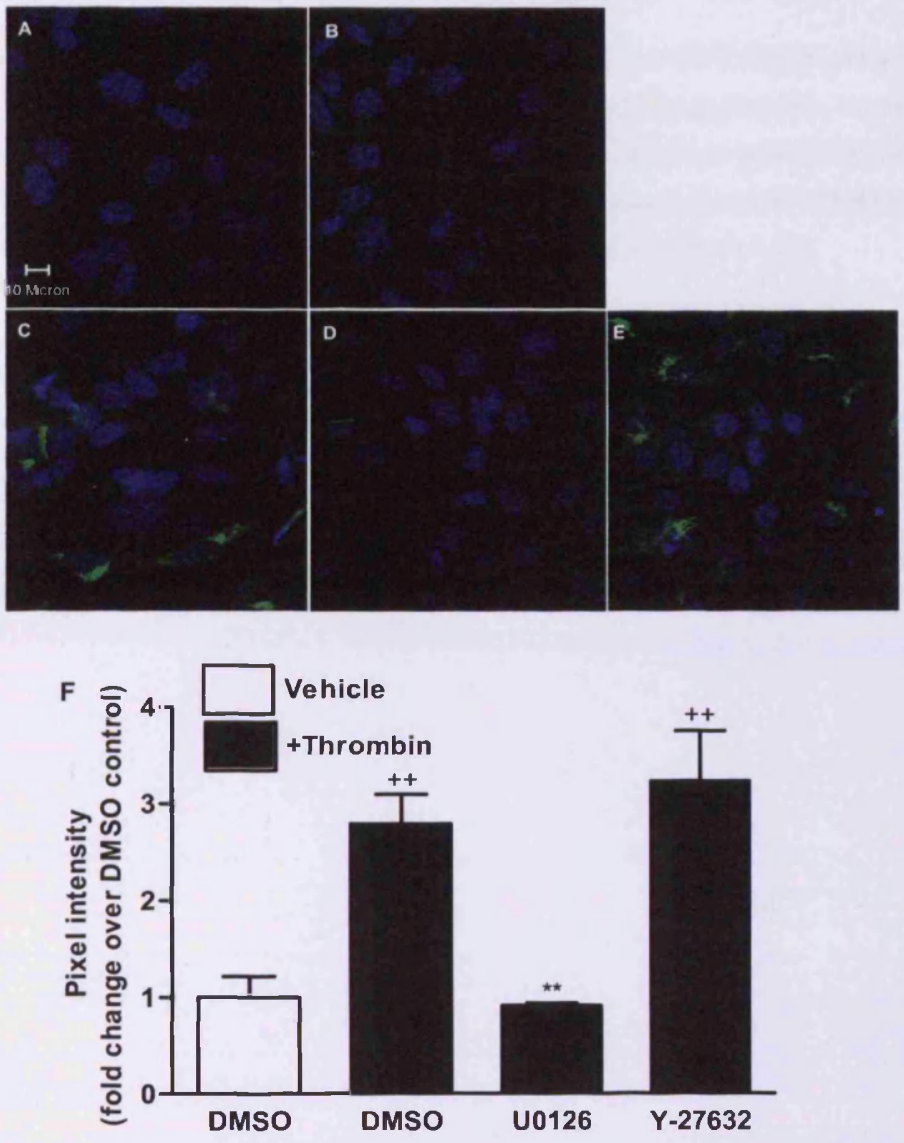


Figure 3.35 Immunocytofluorescence demonstrates that CCL2 intracellular protein production is blocked by MEK1/2 inhibition (U0126) but not by Rho kinase inhibition (Y-27632). Panel A, isotype control. Panel B, untreated cells. Panel C shows the effect of thrombin on CCL2 intracellular protein production. Panel D shows the effect of U0126 on CCL2 intracellular protein production induced by thrombin. Panel E shows the effect of Y-27632 on CCL2 intracellular protein production induced by thrombin. Cells were pre-incubated with or without U0126 (10 μM) or Y-27632 (10 μM) for 30 min before stimulation with thrombin (10 nM) for 3 hours. At the end of incubation, cells were immunostained with normal goat IgG (A) or anti-CCL2 antibody (B-E) and followed by DAPI staining. Areas of CCL2 localization are shown in green, nuclei appear blue. Panel F shows the pixel intensity analysis for each group. Data represent the fold increase over control. Data show the representative of three independent experiments. $^{++}p<0.01$, comparison with untreated cells; $^{**}p<0.01$, comparison with thrombin alone.

In summary, the results in this section show that blockade of ERK1/2 signalling results in near total blockade of thrombin-induced intracellular CCL2 production. In contrast, blockade of Rho kinase signalling is not essential for CCL2 protein production but likely affects the subsequent release of CCL2 from the cell, because thrombin-induced CCL2 levels in cell culture supernatants were inhibited by Y-27632 (Figure 3.30).

Chapter 4: Results (II)

Identification of PAR_1 in mediating thrombin-induced CCL2/MCP-1 release in primary human adult lung fibroblasts

The previous chapter identified the signalling pathways involved in thrombin-induced CCL2 release in mouse lung fibroblasts. Thrombin is also known to induce the expression of CCL2 in primary dermal fibroblasts via the activation of PAR_1 (Bachli *et al.*, 2003), but whether it can do so in primary human lung fibroblasts and the signalling receptor involved are not known. The aim of this chapter was therefore to determine whether thrombin stimulates the production of CCL2 by primary human adult lung fibroblasts (pHALFs), and to identify the signalling receptor (s) involved.

pHALFs were previously isolated in the host laboratory from lung explants (for a more detailed description see Materials and Methods Section 2.8). As they are derived directly from human lung tissue, these cells provide the closet representation of human biology, compared to ordinary immortalized cell lines. In addition, they have been well characterized in our laboratory and have previously been used to establish the effects of thrombin and TGF-β stimulation on fibroblast function (Chambers *et al.*, 2003).

4.1 Effect of thrombin on CCL2 release by primary human adult lung fibroblasts

In order to determine the effect of thrombin on pHALF CCL2 release, time-course experiments were performed with a standard concentration of 10 nM. pHALFs were exposed to 10 nM thrombin for various durations, and CCL2 protein levels in culture supernatants were assessed by ELISA. Figure 4.1A shows that thrombin induced pHALF CCL2 production in a time-dependent manner from 6 hours onwards, and the sharpest increase in CCL2 production occurs over the first 9 hours. In cells without thrombin treatment, there was also baseline production of CCL2 after 12 hour incubation with DMEM. Based on these results, a 6 hour time point was chosen for all subsequent experiments.

For concentration-response experiments, pHALFs were incubated with increasing concentrations of thrombin (0.001 nM to 10 nM) for 6 hours and CCL2 protein levels in culture supernatants were measured by ELISA. Figure 4.1B shows that thrombin stimulated CCL2 production in a concentration-dependent manner from 0.3 nM

onwards. CCL2 production continued to increase at all concentrations examined and the effect did not plateau at the highest concentration of thrombin (10 nM) examined.

4.2 The expression of PARs in primary human adult lung fibroblasts

In order to begin to investigate the involvement of PARs in thrombin-induced CCL2 release in pHALFs, the expression of PAR_{1-4} receptors in pHALFs was first examined. PAR expression was analysed in whole cell lysates by RT-PCR. Figure 4.2 shows that prominent PCR products were obtained for PAR_1, PAR_2 and PAR_3, whereas there was only faint band for PAR_4, suggesting that PAR_4 is expressed at much low levels in these cells compared with PAR_{1-3}.

4.3 The roles of PAR agonists in CCL2 release by primary human adult lung fibroblasts

In order to determine which receptor (s) is/are involved in mediating thrombin-induced CCL2 release by pHALFs, the effect of PAR_1, PAR_2 and PAR_4 activating peptides on pHALF CCL2 production was next examined. The role of PAR_3 in this study was not investigated due to the lack of PAR_3-selective agonist and antagonist, and the lack of evidence that this receptor can signal on its own in fibroblasts.

4.3.1 Effect of PAR_1 agonist peptides TFLLR-NH_2 on CCL2 release by primary human adult lung fibroblasts

In the previous chapter, PAR_1 was shown to be the major receptor involved in thrombin-induced CCL2 release in MLFs, so that the effect of the PAR_1 specific agonist peptide TFLLR on pHALF CCL2 release was examined first. Cells were stimulated with increasing concentrations of TFLLR (6 μM to 200 μM) for 6 hours, and CCL2 protein levels in culture supernatants were assessed by ELISA. As shown in Figure 4.3, the PAR_1 specific activating peptide TFLLR induced pHALF CCL2 production in a concentration-dependent manner from 20 μM onwards. CCL2 production continued to increase at all concentrations and the effect did not plateau at the highest concentration of TFLLR (200 μM) examined. To confirm the specificity of the PAR_1 agonist peptide TFLLR in influencing CCL2 protein levels, the effect of the reverse peptide FTLLR on CCL2 protein levels was also examined in parallel cultures. Figure 4.3 shows that FTLLR significantly induced CCL2 release at 200 μM, suggesting that the stimulatory effect obtained with TFLLR at 200 μM may be non-PAR_1-mediated and non-specific. TFLLR at 60 μM significantly increased CCL2 production, whereas FTLLR at the same concentration had no effect on this response, so that 60 μM was used as a standard concentration for TFLLR for all subsequent studies.

4.3.2 Effect of FXa, SLIGKV (PAR_2 activating peptide) and AYPGKF (PAR_4 activating peptide) on CCL2 release by primary human adult lung fibroblasts

Both PAR_1 and PAR_4 mediate the effect of thrombin in human platelets. PAR_2 is not a thrombin receptor, but it has been reported that PAR_2 can also mediate thrombin's effect after transactivated by PAR_1. As both PAR_2 and PAR_4 are expressed by pHALFs, it is important to investigate the role of PAR_2 and PAR_4 in thrombin-induced CCL2 release by pHALFs. The effect of PAR_2 and PAR_4 activating peptides on pHALF CCL2 release was therefore examined. Both thrombin and TFLLR were used as positive controls. FXa can signal via both PAR_1 and PAR_2, the effect of FXa on pHALF CCL2 release was also examined.

Cells were exposed to thrombin (10 nM), FXa (10 nM), TFLLR (60 μM), SLIGKV (200 μM) and AYPGKF (200 μM) for 6 hours. CCL2 protein levels in culture supernatants were assessed by ELISA. As shown in Figure 4.4, both FXa, and SLIGKV significantly induced pHALF CCL2 release by 5.6 ± 0.7 fold and by 6.6 ± 0.3 fold, respectively. However, both FXa, and SLIGKV were less efficient when compared to the level of induction (8.7 ± 0.7 fold) seen following stimulation with thrombin. TFLLR induced pHALF CCL2 release by 6.9 ± 0.9 fold, but the differences between TFLLR and thrombin were not significant. In contrast, the PAR_4 agonist peptide AYPGKF exerted no effect on CCL2 production, suggesting that PAR_4 is not involved. These data show that both PAR_1 and PAR_2 activation are sufficient for pHALF CCL2 release.

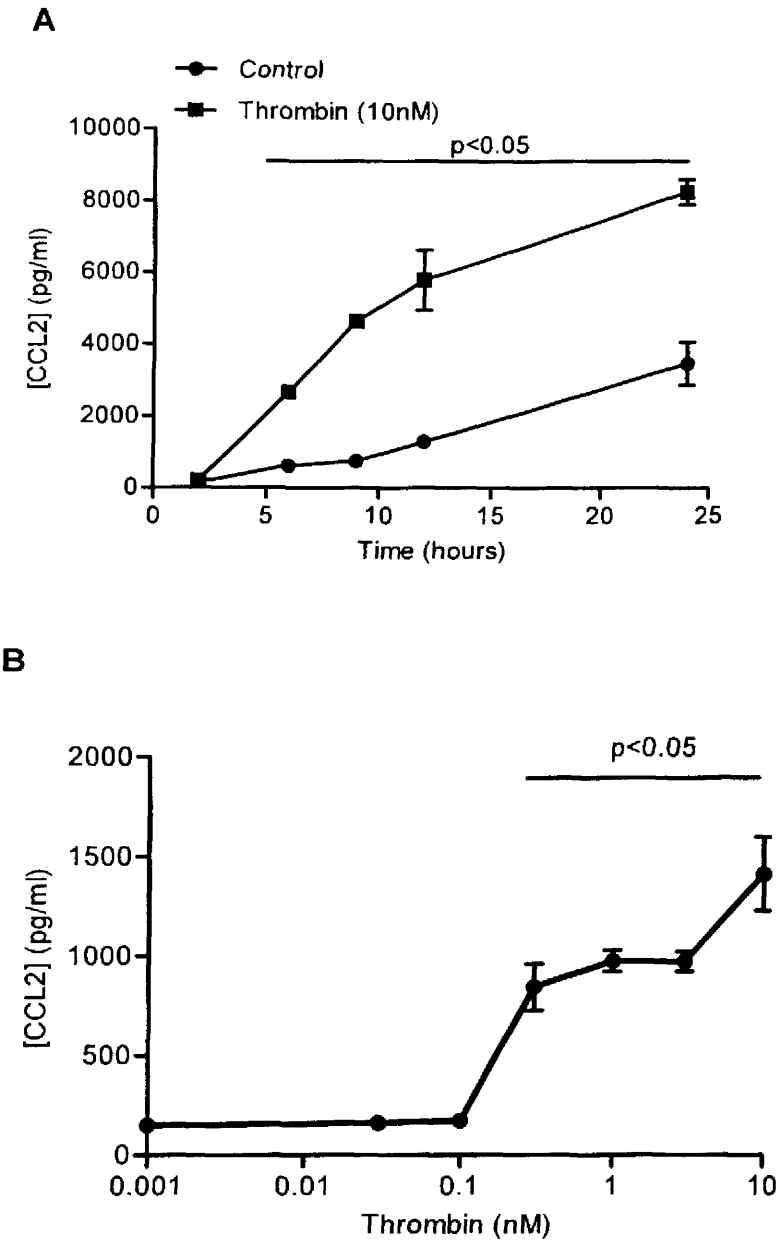


Figure 4.1 Thrombin stimulates pHALF CCL2 protein production in a time- and concentration-dependent manner.

Panels A and B show time-course (A) and concentration response (B) data for the effect of thrombin on pHALF CCL2 protein production. pHALFs were exposed to 10 nM thrombin for varying durations (2 to 24 hours), or exposed to various concentrations of thrombin (0.001 to 10 nM) for 6 hours. Supernatants from cell cultures following incubation were analyzed for CCL2 production by ELISA. The amount of secreted CCL2 is expressed as pg/ml and each value represents the mean ± SEM from triplicates at each condition. Data show representative of three independent experiments. $p<0.05$, comparison with time point-matched media control (panel A), or unstimulated cells (panel B).

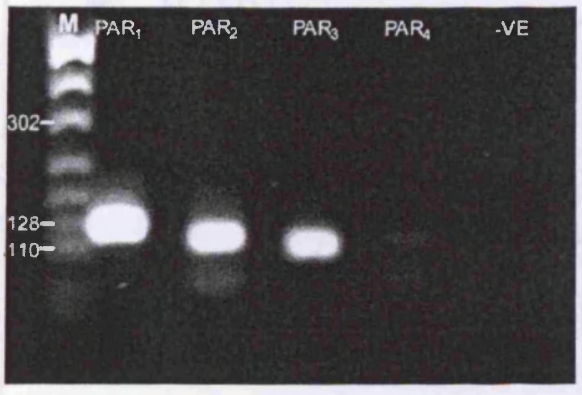


Figure 4.2 Expression of PAR_{1-4} in pHALFs.

Figure shows the expression of PAR_{1-4} as analysed by RT-PCR in whole cell lysates of pHALFs. 1 µg of cDNA was used for each set of primers and the same volume of final PCR products was run in a 1% agarose gel containing ethidium bromide. pHALFs show expression of PAR_1, PAR_2, PAR_3, and much low level of PAR_4. PAR_1 primers amplify a 148bp product; PAR_2 primers amplify a 128bp product; PAR_3 primers amplify a 111bp product and PAR_4 primers amplify a 119bp product. –VE, negative control. Data show representative of three independent experiments.

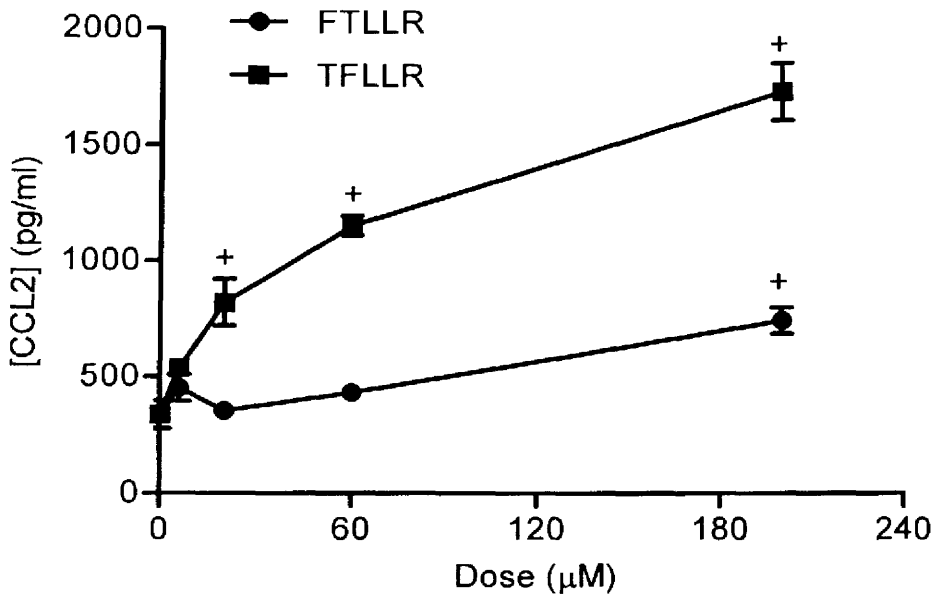


Figure 4.3 Effect of TFLLR and the reverse control peptide FTLLR on CCL2 production by primary human adult lung fibroblasts.

Panel A and B show the effects of TFLLR and FTLLR on pHALF CCL2 production. pHALFs were incubated with various concentrations of TFLLR and FTLLR (6 μM to 200 μM) for 6 hours. Supernatants from cell cultures following incubation were analyzed for CCL2 production by ELISA. The amount of secreted CCL2 is expressed as pg/ml and each value represents the mean ± SEM from triplicate wells for each condition. Data show representative of three independent experiments. $^{+}p<0.05$, comparison with unstimulated cells.

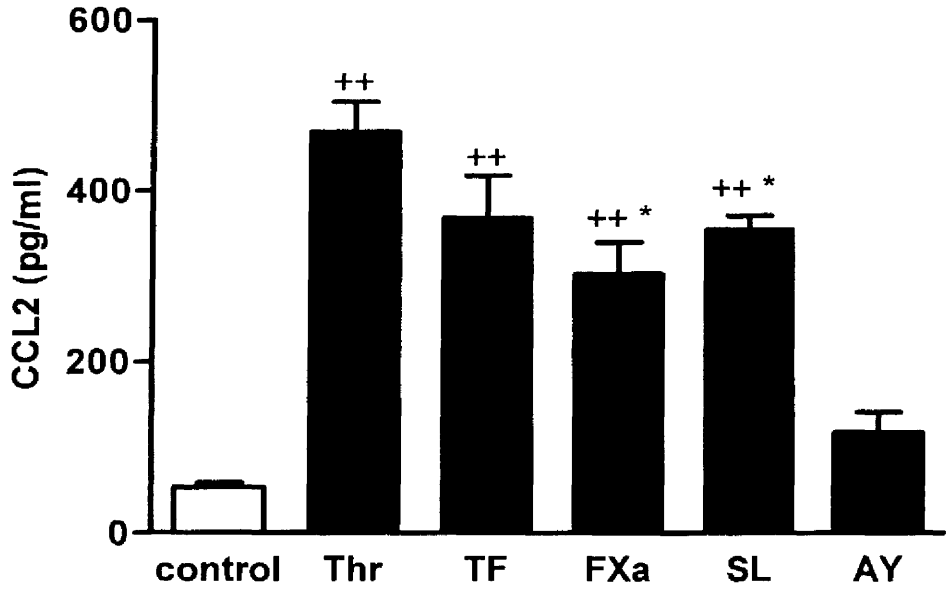


Figure 4.4 Effect of thrombin, TFLLR, FXa, SLIGKV, and AYPGKF on CCL2 production by primary human adult lung fibroblasts.

Panel shows the effect of PAR_1, PAR_2 and PAR_4 agonist peptides on CCL2 production by primary human adult lung fibroblasts. pHALFs were exposed to thrombin (Thr, 10 nM), FXa (10 nM), TFLLR (TF, 60 μM), SLIGKV (SL, 200 μM) and AYPGKF (AY, 200 μM) for 6 hours. Supernatants from cell cultures following incubation were analyzed for CCL2 production by ELISA. The amount of secreted CCL2 is expressed as pg/ml and each value represents the mean ± SEM from triplicates at each concentration. Data show representative of three independent experiments. $^{++}p<0.01$, comparison with unstimulated cells; $^{*}p<0.05$, comparison with thrombin.

4.4 Summary

The results described in this section examining the effects of PAR agonists on pHALF CCL2 production showed that:

- Thrombin induces pHALF CCL2 production in a concentration-dependent manner from 0.3 nM onwards.
- Thrombin (10 nM) induces pHALF CCL2 production in a time-dependent manner from 6 hours onwards, and the sharpest increase in CCL2 production occurs over the first 9 hours.
- PAR_1 is strongly expressed in pHALF; PAR_2 and PAR_3 are also expressed, whereas PAR_4 is expressed at much lower levels.
- PAR_1 specific agonist peptide TFLLR can mimic the effect of thrombin on CCL2 release in pHALFs, suggesting that PAR_1 is sufficient for pHALF CCL2 release.
- Activation of PAR_2 by the PAR_2 activating peptide SLIGKV induces pHALF CCL2 production.
- The PAR_1 and PAR_2 activator FXa stimulates significant pHALF CCL2 release, but is less efficient than thrombin at equivalent concentrations.
- PAR_4 agonist peptide AYPGKF did not induce pHALF CCL2 release, suggesting that PAR_4 is not involved.

In conclusion, both PAR_1 and PAR_2 activation are sufficient for CCL2 production by pHALFs.

4.5 Role of PAR_1 activation in CCL2 release by primary human adult lung fibroblasts

4.5.1 Effect of RWJ-58259 on thrombin (10 nM)-induced CCL2 release by primary human adult lung fibroblasts

The previous chapter showed that PAR_1 is the major receptor involved in mediating thrombin-induced CCL2 release in MLFs at 10 nM of the proteinase. In order to examine whether this is also the case for pHALFs, the effect of PAR_1 specific antagonist RWJ-58259 on this response was next examined. Cells were pre-incubated with various concentrations (0.01 μM to 3 μM) of the PAR_1 specific antagonist RWJ-58259 for 15 minutes before exposure to thrombin (10 nM) for 6 hours, and CCL2 protein levels in culture supernatants were assessed by ELISA. Surprisingly, unlike the results obtained for MLFs, RWJ-58259 did not inhibit thrombin-induced CCL2 release in pHALFs (Figure 4.5A). On the contrary, RWJ-58259 at concentrations higher than 1 μM increased both basal and thrombin-induced CCL2 release in pHALFs (Figure 4.5 B).

The lack of an effect of RWJ-58259 might be explained by insufficient inhibition of PAR_1 activation. These data can also be interpreted as indicating that PAR_1 might not be involved in mediating thrombin (10 nM)-induced CCL2 release in pHALFs. In order to investigate these possibilities further, the effect of RWJ-58259 on PAR_1 agonist peptide TFLLR, and the $PAR_{1/2}$ activator FXa-induced pHALF CCL2 release was next examined.

4.5.2 Effect of RWJ-58259 on TFLLR and FXa-induced CCL2 release by primary human adult lung fibroblasts

Cells were pre-incubated with various concentrations of the PAR_1 specific antagonist RWJ-58259 for 15 minutes before exposure to TFLLR (60 μM) for 6 hours, and CCL2 protein levels in culture supernatants were assessed by ELISA. As shown in Figure 4.6, RWJ-58259 significantly inhibited TFLLR-induced CCL2 release in a concentration-dependent manner from 0.1 μM onwards. At 1 μM (-Log [1 μM]=6), RWJ-58259 inhibited this response by 72 ± 8% ($p<0.05$). These data indicate that PAR_1 is both sufficient and necessary for TFLLR-induced CCL2 release by pHALFs.

The effect of RWJ-58259 on FXa-induced pHALF CCL2 release was next examined. Cells were pre-incubated with various concentrations of the PAR_1 specific antagonist RWJ-58259 for 15 minutes before exposure to FXa (10 nM) for 6 hours. Figure 4.7A shows that RWJ-58259 inhibited FXa-induced CCL2 production in pHALFs in a

concentration dependent-manner from 0.1 μM onwards. At 0.1 μM (-Log [0.1 μM]=7), RWJ-58259 inhibited this response of FXa by 79 ± 6% ($p<0.05$), but had no effect on basal CCl2 production. However, only 54% inhibition of FXa-induced CCL2 production was obtained with RWJ-58259 at 1 μM. This may be explained by the increase in basal CCL2 production induced by RWJ-58259 at 1 μM which is sometimes observed in pHALFs (Figure 4.7B). These data suggest that PAR_1 is the major signalling receptor involved in this response of FXa.

Inhibition of both TFLLR and FXa-induced pHALF CCL2 release by RWJ-58259 suggests that the lack of an effect of PAR_1 inhibition on thrombin-induced pHALF CCL2 release may be interpreted as indicating that PAR_1 is not necessary for mediating this response of thrombin in pHALFs. Previous studies have demonstrated that cleaved PAR_1 is able to mediate thrombin's cellular response indirectly via the transactivation of PAR_2 (O'Brien et al 2000). It is therefore possible that thrombin might act via the transactivation of PAR_2 to induce CCL2 production in pHALFs when PAR_1 activation is inhibited by RWJ-58259 which blocks the receptor activation by binding the second extracellular loop but does not prevent unmaking of the tethered ligand. Further experiments were performed to investigate this possibility.

4.5.3 Effect of PAR_1 specific antibodies ATAP2 and WEDE15 on thrombin (10 nM)-induced CCL2 release by primary human adult lung fibroblasts

To further investigate the participation of PAR_1 in thrombin-induced CCL2 release in pHALFs, two previously developed monoclonal antibodies which block PAR_1 cleavage by thrombin were employed. The first antibody, ATAP2, binds to an epitope within the PAR_1 tethered ligand domain (Brass *et al.*, 1994), whereas the second antibody, WEDE15, binds to the hirudin-like domain that interacts with thrombin's anion-binding exosite (Hoxie *et al.*, 1993). Cells were pre-incubated with ATAP2, WEDE15, or the combination of both ATAP2 and WEDE15 (25 μg/ml each) for 30 minutes before exposure to thrombin (10 nM) for 6 hours. Pre-incubation time and concentration of both ATAP2 and WEDE15 were chosen according to previous studies performed by O'Brien and colleagues (O'Brien *et al.*, 2000). This study also reported that neither antibody was sufficient on its own to completely prevent PAR_1 signalling; whereas complete blockade occurred only when the two antibodies were added together.

As shown in Figure 4.8, the combination of ATAP2 and WEDE15 had little effect on basal CCL2 production. Both ATAP2 and WEDE15 antibodies significantly inhibited thrombin-induced pHALF CCL2 release by 48.2 ± 7% ($p<0.05$) and 27.6 ± 1% ($p<0.05$),

respectively. These data suggest that PAR_1 cleavage is necessary for thrombin-induced CCL2 production in pHALFs. The combination of ATAP2 and WEDE15 blocked thrombin-induced CCL2 release by 67.1 ± 7%. This further inhibition was significant when compared with the induction obtained with ATAP2 or WEDE15 alone. These data suggest that PAR_1 is at least in part, responsible for thrombin-induced CCL2 release in pHALFs.

Although it has been reported that PAR_1 can transactivate PAR_2 for mediating thrombin's effect when PAR_1 activation is inhibited, PAR_1 cleavage is required in order to transactivate PAR_2 by its free tethered ligand. It has previously been reported that complete blockade of PAR_1 cleavage by thrombin occurs when both ATAP2 and WEDE15 are added together (O'Brien *et al.*, 2000). However, complete inhibition of CCL2 production was not obtained with the combination of ATAP2 and WEDE15 in this study. Taken together, these data therefore suggest that up to 70% of thrombin-induced CCL2 release is PAR_1-mediated, and the remaining 30% being mediated via a PAR_1-independent mechanism in pHALFs.

4.5.4 Effect of Q94, U0126 and Y-27632 on thrombin (10 nM)-induced CCL2 release by primary human adult lung fibroblasts

Previous results obtained with MLFs showed that PAR_1 coupling to $G\alpha_q$ and downstream ERK1/2 and Rho kinase pathways mediate thrombin (10 nM)-induced CCL2 production. To further investigate the signalling pathways involved in thrombin (10 nM)-induced CCL2 production by pHALFs, the role of $G\alpha_q$, ERK1/2 and Rho kinase was next examined. Cells were treated with increasing concentrations of Q94 (Figure 4.9A), U0126 (Figure 4.9B) and Y-27632 (Figure 4.9C) for 30 minutes before exposure to thrombin (0.3 nM) for 6 hours. Figure 4.9 shows that all three inhibitors attenuated thrombin-induced pHALF CCL2 production in a concentration dependent manner. At 10 μM, U0126 and Y-27632 inhibited this effect of thrombin by 74 ± 2.4% and 80 ± 6.9%, respectively. In contrast, the maximum inhibition obtained with Q94 was 53 ± 5%. These data confirm that PAR_1 is a major signalling receptor involved in thrombin-induced CCL2 release in pHALFs, but that not all of thrombin's effects are mediated via PAR_1. In contrast, both the ERK1/2 and Rho kinase signalling pathways account for most of thrombin-induced CCL2 release by pHALFs.

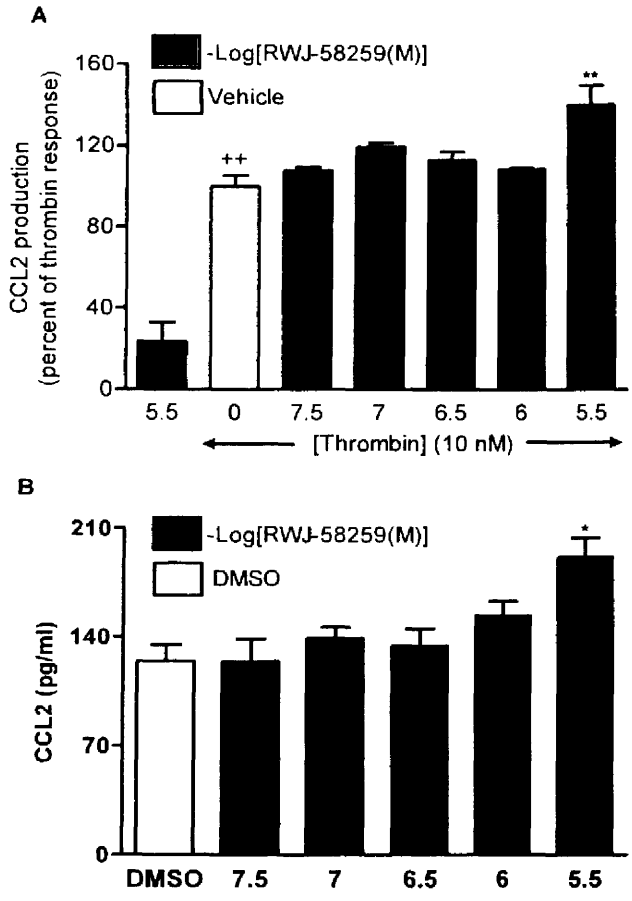


Figure 4.5 Inhibition of PAR_1 by RWJ-58259 does not inhibit thrombin (10 nM)-induced CCL2 release by primary human adult lung fibroblasts

Panel A shows the effect of the PAR_1 antagonist RWJ-58259 on pHALF CCL2 protein production in response to 10 nM thrombin. Data are presented as a percentage of the maximal response obtained with thrombin and drug vehicle alone (0.03% DMSO in DMEM). The first bar represents the highest concentration of RWJ-58259 which increased the basal CCL2 release (see panel B). Panel B shows the effect of RWJ-58259 on basal CCL2 production in pHALFs, and the amount of secreted CCL2 is expressed as pg/ml. Cells were treated with increasing concentrations of RWJ-58259 for 15 minutes before stimulation with thrombin for 6 hours. Final concentrations of DMSO were kept constant for all treatment conditions. Negative log of the concentrations of RWJ-58259 are presented. Data represent the mean ± SEM of triplicates, and show representative of three independent experiments. $^{++}p<0.01$, comparison with unstimulated cells; $^{**}p<0.01$, comparison with thrombin; $^{*}p<0.05$, comparison with DMSO control.

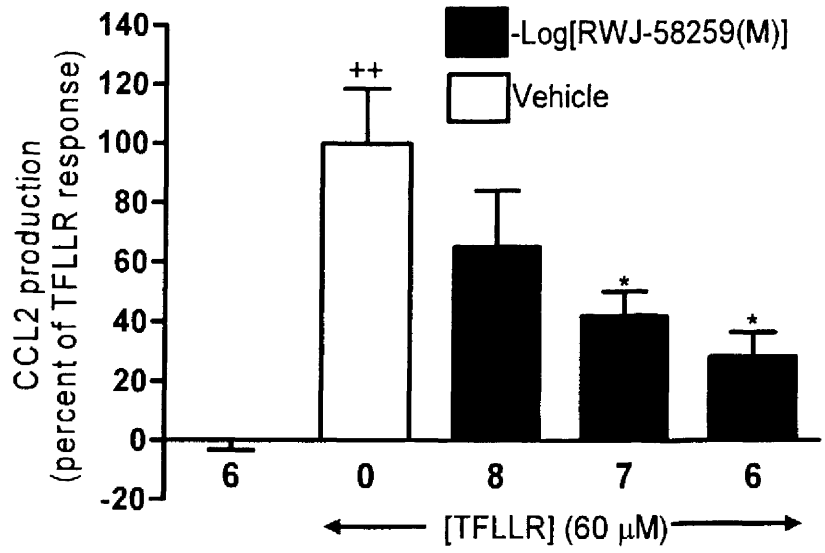


Figure 4.6 PAR_1 is necessary and sufficient for TFLLR-induced CCL2 release by primary human adult lung fibroblasts

Panel shows the effect of the PAR_1 antagonist RWJ-58259 on pHALF CCL2 release in response to TFLLR (60 μM). Data are presented as a percentage of the maximal response obtained with TFLLR and drug vehicle alone (0.01% DMSO in DMEM). Cells were treated with increasing concentrations of RWJ-58259 for 15 minutes before stimulation with TFLLR for 6 hours. Final concentrations of DMSO were kept constant for all treatment conditions. The first bar represents the highest concentration of RWJ-58259 used and shows that this compound has no effect on basal CCL2 production. Negative log of the concentrations of RWJ-58259 are presented. Data represent the mean ± SEM of triplicates, and show representative of three independent experiments. $^{++}p<0.01$, comparison with unstimulated cells; $^{*}p<0.05$, comparison with TFLLR.

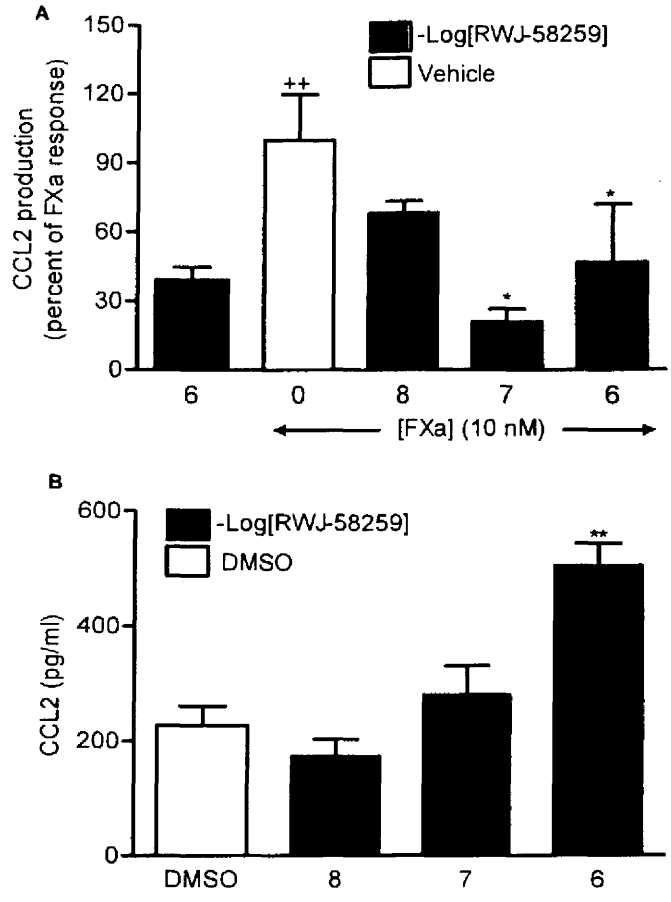


Figure 4.7 PAR_1 is necessary for FXa-induced CCL2 release by primary human adult lung fibroblasts

Panel A shows the effect of the PAR_1 antagonist RWJ-58259 on pHALF CCL2 release in response to FXa (10 nM). Data are presented as a percentage of the maximal response obtained with FXa and drug vehicle alone (0.01% DMSO in DMEM). The first bar represents the highest concentration of RWJ-58259 which increased the basal CCL2 release (see panel B). Panel B shows the effect of RWJ-58259 on basal CCL2 production in pHALFs, and the amount of secreted CCL2 is expressed as pg/ml. Cells were treated with increasing concentrations of RWJ-58259 for 15 minutes before stimulation with FXa for 6 hours. Final concentrations of DMSO were kept constant for all treatment conditions. Negative log of the concentrations of RWJ-58259 are presented. Data represent the mean ± SEM of triplicates, and show representative of three independent experiments. $^{++}p<0.01$, comparison with unstimulated cells; $^{*}p<0.05$, comparison with thrombin. $^{**}p<0.01$, comparison with DMSO control.

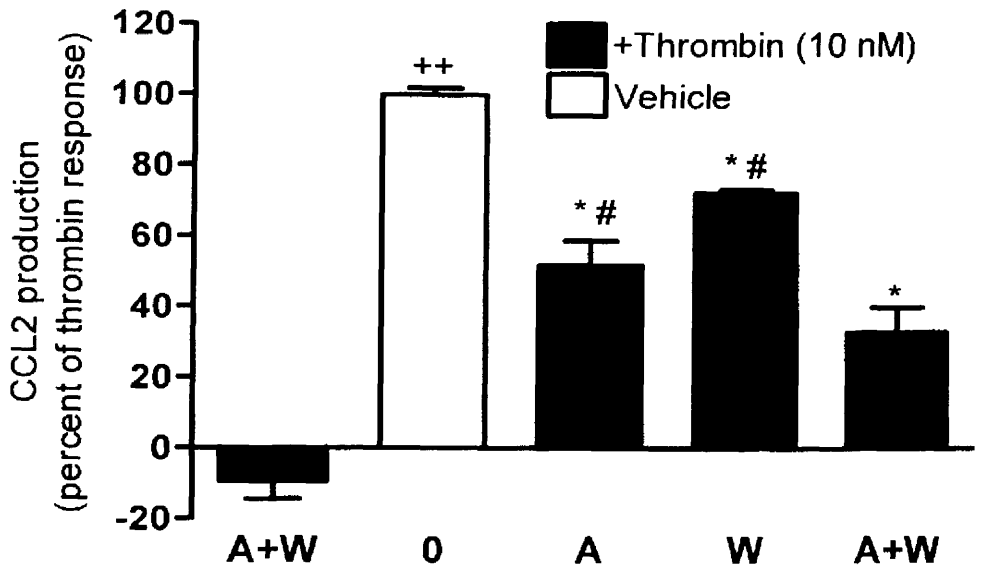


Figure 4.8 PAR_1 is partially involved in thrombin (10 nM)-induced CCL2 release by primary human adult lung fibroblasts

Panel shows the effect of the PAR_1 antibodies (ATAP2 and WEDE15) on pHALF CCL2 release in response to 10 nM thrombin. Data are presented as a percentage of the maximal response obtained with thrombin and drug vehicle alone. Cells were incubated with ATAP2 (A), WEDE15 (W), or with both ATAP2 and WEDE15 (A+W) for 30 minutes before exposure to thrombin for 6 hours. The concentration used for both antibodies is 25 µg/ml. The first bar represents the group of cells treated with ATAP2 and WEDE15 only, and shows that these antibodies have no significant effect on basal CCL2 release. Data represent the mean ± SEM of triplicates, and show representative of three independent experiments. $^{++}p<0.01$, comparison with unstimulated cells; $^{*}p<0.05$, comparison with thrombin; $^{\#}p<0.05$, comparison with cells treated with both antibodies and thrombin.

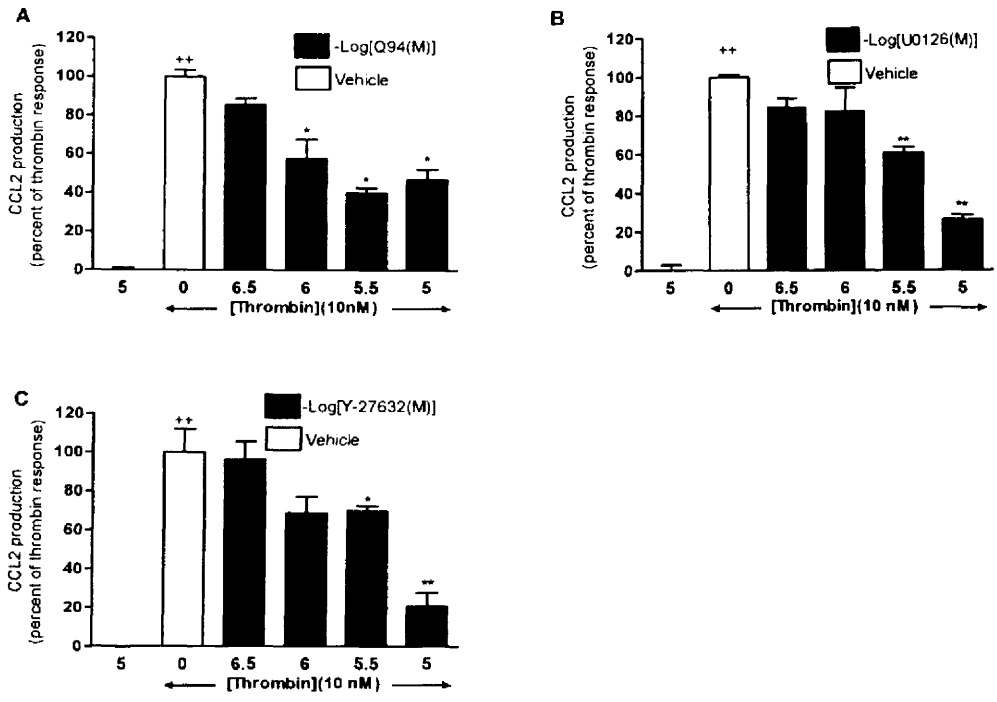


Figure 4.9 Inhibition of $G\alpha_q$ (Q94), MEK1/2 (U0126) and Rho kinase (Y-27632) attenuates thrombin (10nM)-induced pHALF CCL2 release.

Figure shows the effects of Q94, U0126 and Y-27632 on thrombin-induced CCL2 release. Cells were pre-incubated with increasing concentrations of inhibitors for 30 minutes before exposure to thrombin (10 nM) for 6 hours. Data are presented as a percentage of the maximal response obtained with thrombin and drug vehicle alone (0.1% DMSO in DMEM). Final concentrations of DMSO were kept constant for all treatment conditions. The first bars represent the highest concentrations of inhibitors used, and show that these compounds have no significant effect on basal CCL2 production. Negative log of the concentrations of inhibitors are presented. Data represent the mean ± SEM from triplicates, and show representative of three independent experiments. $^{++}p<0.01$, comparison with medium control; $^{*}p<0.05$, comparison with thrombin alone; $^{**}p<0.01$, comparison with thrombin.

4.6 Summary

The results described in this section exploring the role of PAR_1 on pHALF CCL2 release induced by thrombin (10 nM) showed that:

- Inhibition of PAR_1 by RWJ-58259 inhibited both TFLLR- and FXa-induced pHALF CCL2 release in a concentration-dependent manner, suggesting that PAR_1 activation is sufficient for CCL2 release in pHALFs.

- Inhibition of PAR_1 by RWJ-58259 did not inhibit pHALF CCL2 release induced by thrombin at a standard concentration of 10 nM. In contrast, the $G\alpha_q$ selective PAR_1 antagonist Q94 blocked this response of thrombin by about 50%, suggesting that PAR_1 is partially involved in mediating thrombin-induced CCL2 release in pHALFs.

- Inhibition of PAR_1 cleavage by ATAP2 or WEDE15 inhibited 10 nM thrombin-induced pHALF CCL2 release by 48.2 ± 7% and 27.6 ± 1% respectively, suggesting that PAR_1 is necessary for pHALF CCL2 release induced by thrombin.

- Inhibition of PAR_1 cleavage by both ATAP2 and WEDE15 did not fully block this effect of thrombin (67.1 ± 7%). It has been reported that complete inhibition of PAR_1 cleavage is obtained with the combination of ATAP2 and WEDE15. These data therefore suggest that PAR_1-independent mechanism may also be involved.

- Inhibition of ERK1/2 and Rho kinase inhibited thrombin-induced pHALF CCL2 release in a concentration-dependent manner by up to 74 ± 2.4% and 80 ± 6.9%, respectively, suggesting that both ERK1/2 and Rho kinase signalling pathways account for most of thrombin-induced CCL2 release.

Taken together, these data suggest that thrombin (10 nM) induces pHALF CCL2 protein release via both PAR_1-dependent and PAR_1-independent mechanisms. PAR_1 coupling to $G\alpha_q$ is partially involved, whereas both ERK1/2 and Rho kinase signalling pathways account for most of thrombin-induced pHALF CCL2 release.

4.7 The role of PAR_1 in low concentration (0.3 nM) of thrombin-induced CCL2 release by primary human adult lung fibroblasts

The data obtained thus far suggested that thrombin-induced CCL2 release by pHALFs at 10 nM is partially PAR_1-dependent. Extensive studies by other investigators have demonstrated that thrombin at different concentrations may signal via different PAR receptors to mediate their downstream cellular responses (Reviewed in (O'Brien *et al.*, 2001)). The data presented in figure 4.1B show that thrombin induces CCL2 release in a concentration-dependent manner from 0.3 nM onwards. To determine the relative contribution of PAR_1 to thrombin-induced CCL2 release at lower concentrations, the role of PAR_1 in 0.3 nM thrombin-induced pHALF CCL2 release was examined.

4.7.1 Effect of RWJ-58259 on low concentration thrombin (0.3 nM)-induced CCL2 release by primary human adult lung fibroblasts

In order to investigate the role of PAR_1 in CCL2 production in response to low concentration of thrombin (0.3 nM) in pHALFs, the effect of RWJ-58259 on this response was examined. Cells were pre-incubated with various concentrations of the PAR_1 specific antagonist RWJ-58259 for 15 minutes before exposure to thrombin (0.3 nM) for 6 hours. The highest concentration of RWJ-58259 used was 1 μM because previous experiments demonstrated that RWJ-58259 affects basal CCL2 production at higher concentrations (see Figure 4.5). Figure 4.10 shows that RWJ-58259 significantly inhibited thrombin-induced CCL2 release in a concentration-dependent manner from 0.1 μM onwards. At 0.3 μM (-log [0.3 μM]=6.5), RWJ-58259 inhibited this response by 76 ± 6.5% ($p<0.01$). These data suggest that PAR_1 is necessary for mediating CCL2 release induced by thrombin at low concentrations of the proteinase (0.3 nM).

4.7.2 Effect of ATAP2 and WEDE15 on low concentration thrombin (0.3 nM)-induced CCL2 release by primary human adult lung fibroblasts

In order to further investigate the role of PAR_1 in mediating low concentration of thrombin (0.3 nM)-induced CCL2 release in pHALFs, the effect of the two PAR_1-specific monoclonal antibodies, ATAP2 and WEDE15, on low concentration of thrombin (0.3 nM)-induced CCL2 release was examined. Cells were pre-incubated with ATAP2, WEDE15, or the combination of both ATAP2 and WEDE15 (25 μg/ml each) for 30 minutes. Cells were then stimulated with thrombin (0.3 nM) for 6 hours. Figure 4.11 shows both ATAP2 and WEDE15 significantly inhibited thrombin-induced CCL2 release by 83 ± 10% ($p<0.01$) and by 77 ± 3% ($p<0.01$), respectively. The combination of ATAP2 and WEDE15 blocked thrombin-induced CCL2 release by 93 ± 1.3% ($p<0.01$).

4.7.3 Effect of Q94, U0126 and Y-27632 on low concentration thrombin (0.3 nM)-induced CCL2 release by primary human adult lung fibroblasts

To determine the role of $G\alpha_q$, ERK1/2 and Rho kinase in low concentration of thrombin (0.3 nM)-induced pHALF CCL2 release, the effect of Q94, U0126 and Y-27632 on this response was next examined. Cells were treated with increasing concentrations of Q94 (Figure 4.12A), U0126 (Figure 4.12B) and Y-27632 (Figure 4.12C) before exposure to thrombin (0.3 nM) for 6 hours. Figure 4.12 shows that all three inhibitors blocked thrombin-induced pHALF CCL2 production in a concentration-dependent manner. At 10 μM (-log [10 μM]=5), Q94, U0126, and Y-27632 inhibited this response of thrombin by 86.7 ± 7.5%, 85.5 ± 2% and 89.3 ± 2% (all $p<0.01$), respectively. These data suggest that PAR_1-$G\alpha_q$, ERK1/2 and Rho kinase signalling pathways mediate thrombin-induced CCL2 release by pHALFs at 0.3 nM of this proteinase. In contrast, previous results showed that PAR_1-$G\alpha_q$ is not fully involved in thrombin-induced CCL2 release in pHALFs at 10 nM. This discrepancy is consistent with the notion that thrombin at different concentrations may signal via different mechanism to mediate its downstream cellular responses.

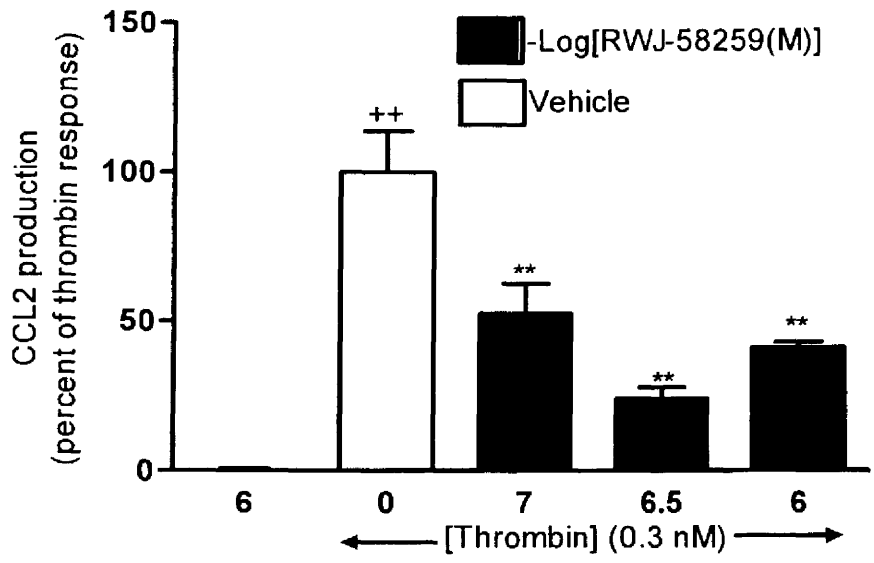


Figure 4.10 PAR_1 is necessary and sufficient for thrombin-induced CCL2 release by primary human adult lung fibroblasts at low concentrations of the proteinase (0.3 nM). Panel shows the effect of the PAR_1 antagonist RWJ-58259 on pHALF CCL2 release in response to 0.3 nM thrombin. Data are presented as a percentage of the maximal response obtained with thrombin and drug vehicle alone (0.01% DMSO in DMEM). Cells were treated with increasing concentrations of RWJ-58259 for 15 minutes before stimulation with thrombin for 6 hours. Final concentrations of DMSO were kept constant for all treatment conditions. The first bar represents the highest concentration of RWJ-58259 used and shows that this compound has no effect on basal CCL2 production. Negative log of the concentrations of RWJ-58259 are presented. Data represent the mean ± SEM of triplicates, and show representative of three independent experiments. $^{++}p<0.01$, comparison with unstimulated cells; $^{**}p<0.01$, comparison with thrombin.

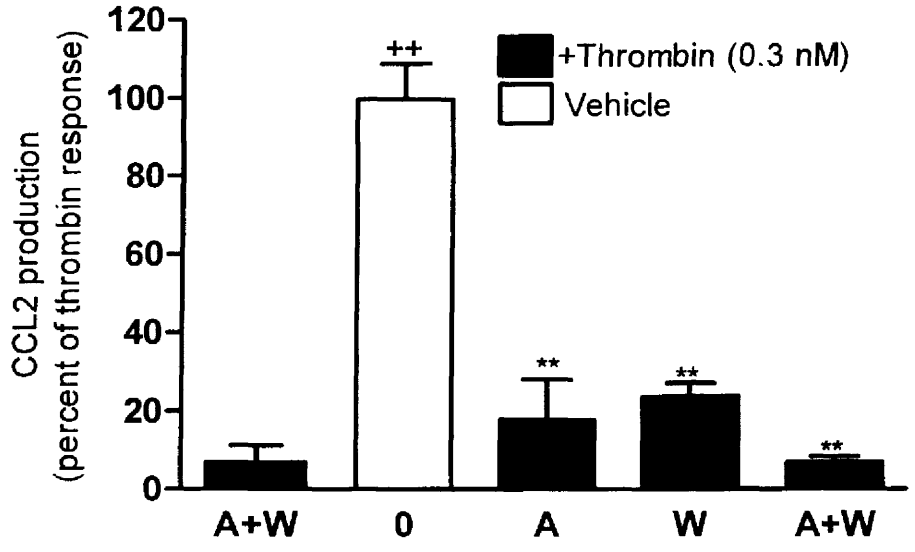


Figure 4.11 PAR_1 is sufficient and necessary for mediating thrombin (0.3 nM)-induced CCL2 release by primary human adult lung fibroblasts

Panel shows the effect of the PAR_1 blocking antibodies (ATATP2 and WEDE15) on pHALF CCL2 release in response to thrombin at 0.3 nM. Data are presented as a percentage of the maximal response obtained with thrombin and drug vehicle alone. Cells were treated with ATAP2 (A), WEDE15 (W), or with both ATAP2 and WEDE15 (A+W) for 30 minutes before stimulation with thrombin for 6 hours. The concentration used for both antibodies is 25 μg/ml. The first bar represents the group of cells treated with ATAP2 and WEDE15 only, and shows that this antibody mixture has no significant effect on basal CCL2 production. Data represent the mean ± SEM of triplicates, and show representative of three independent experiments. $^{++}p<0.01$, comparison with unstimulated cells; $^{**}p<0.01$, comparison with thrombin.

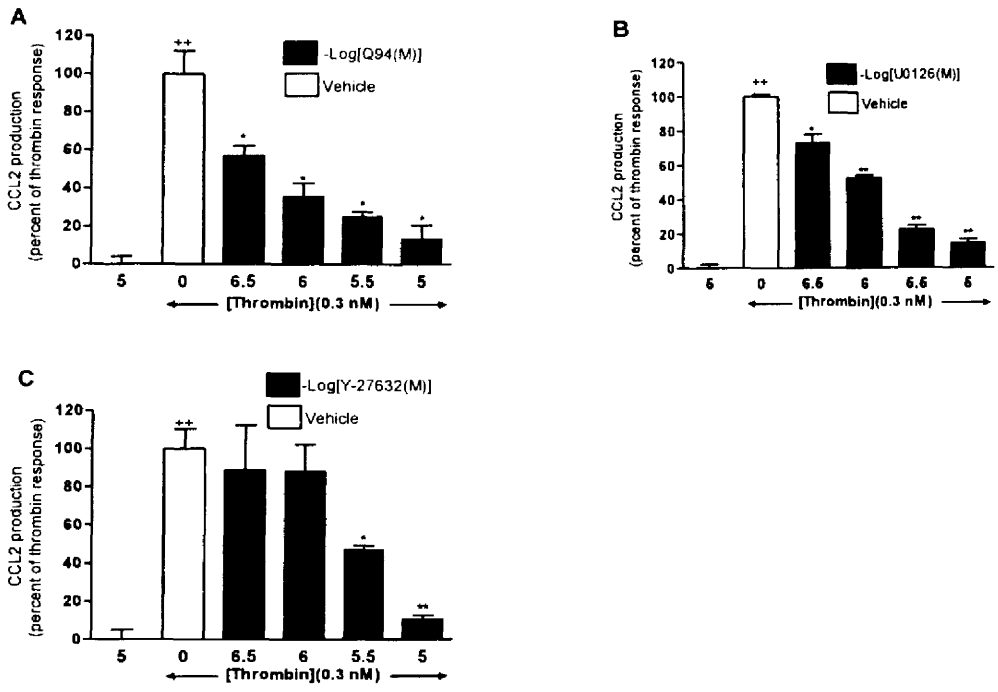


Figure 4.12 Inhibition of $G\alpha_q$ (Q94), and MEK1/2 (U0126) and Rho kinase (Y-27632) attenuates thrombin (0.3 nM)-induced pHALF CCL2 release.

Figure shows the effects of Q94, U0126 and Y-27632 on thrombin-induced CCL2 release. Cells were pre-incubated with increasing concentrations of inhibitors for 30 minutes before exposure to thrombin (0.3 nM) for 6 hours. Data are presented as a percentage of the maximal response obtained with thrombin and drug vehicle alone (0.1% DMSO in DMEM). Final concentrations of DMSO were kept constant for all treatment conditions. The first bars represent the highest concentrations of inhibitors used, and show that these compounds have no significant effect on basal CCL2 production. Negative log of the concentrations of inhibitors are presented. Data represent the mean ± SEM from triplicates, and show representative of three independent experiments. $^{++}p<0.01$, comparison with medium control; $^{*}p<0.05$ and $^{**}p<0.01$, comparison with thrombin.

4.8 Summary

The results described in this section exploring the role of PAR_1 on pHALF CCL2 release induced by thrombin at low concentration (0.3 nM) showed that:

- 0.3 nM thrombin-induced pHALF CCL2 release was inhibited by RWJ-58259 in a concentration-dependent manner, suggesting that PAR_1 is necessary for mediating this response.

- Inhibition of PAR_1 by ATAP2, WEDE15 or both ATAP2 and WEDE15, completely inhibited 0.3 nM thrombin-induced pHALF CCL2 release, further suggesting that the effect of 0.3 nM thrombin on pHALF CCL2 release is mediated by the activation of PAR_1.

- Inhibition of PAR_1 coupling to $G\alpha_q$, MEK1/2, and Rho kinase inhibited thrombin-induced pHALF CCL2 production in a concentration-dependent manner. Full blockade was obtained at highest concentrations of all inhibitors, suggesting that $G\alpha_q$, ERK1/2, and Rho kinase play major roles in this response of thrombin at 0.3 nM.

In conclusion, thrombin (0.3 nM)-induced pHALF CCL2 release is mediated by PAR_1 coupling to $G\alpha_q$, ERK1/2 and Rho kinase signalling pathways.

Chapter 5: Discussion

Overview

The activation of the coagulation cascade leading to the generation of thrombin is one of the earliest events following tissue injury. PAR_1, the main high-affinity thrombin signalling receptor, contributes to excessive inflammation and tissue remodelling in a number of disease settings, including atherosclerosis (Major *et al.*, 2003), vascular neointima formation (Cheung *et al.*, 1999), glomerulonephritis (Cunningham *et al.*, 2000), liver fibrosis (Fiorucci *et al.*, 2004), inflammatory bowel disease (Vergnolle *et al.*, 2004), acute lung injury (Jenkins *et al.*, 2006) and fibrotic lung disease (Howell *et al.*, 2005). In this setting, we have recently shown that the dramatic attenuation of the acute inflammatory and late fibrotic response in PAR_1-deficient mice following bleomycin-induced lung injury is associated with a marked reduction in pulmonary levels of CCL2 (Howell *et al.*, 2005). CCL2 has been shown to play an important role in pulmonary fibrosis as a pro-inflammatory and pro-fibrotic chemokine (Zhang *et al.*, 1994; Moore *et al.*, 2001; Gharaee-Kermani *et al.*, 2003; Inoshima *et al.*, 2004). Delineation of the signalling pathways downstream of PAR_1 activation which lead to CCL2 production may identify multiple novel therapeutic targets for respiratory conditions associated with excessive coagulation proteinase signalling.

The aim of this study was to further our understanding of the signal transduction mechanisms underlying thrombin-induced CCL2 production. Thrombin has been shown to induce CCL2 production in various cell types, such as human glomerular mesangial cells (Grandaliano *et al.*, 1994), human proximal tubular cells (Grandaliano *et al.*, 2000), macrophages (Szaba and Smiley, 2002), dermal fibroblasts (Bachli *et al.*, 2003) and stellate cells (Fiorucci *et al.*, 2004) etc. To the best of my knowledge, the present study is the first to examine the effect of thrombin on the expression of CCL2 and to delineate the signal transduction mechanisms for CCL2 production in mouse lung fibroblasts (MLFs) and in primary human lung fibroblasts (pHALFs), the major cell type responsible for progressive lung fibrosis in animal models and in patients with pulmonary fibrosis. In this study, by using a novel PAR_1-$G\alpha_q$ antagonist which blocks the interaction between PAR_1 and $G\alpha_q$, we report for the first time that PAR_1 coupling to $G\alpha_q$ is essential for thrombin-induced CCL2 gene expression and protein release in MLFs. The studies further demonstrate that these effects are mediated via the co-operation between ERK1/2 and Rho kinase signalling pathways: a calcium-independent PKC, c-Raf and ERK1/2 pathway was found to mediate PAR_1-induced

CCL2 gene transcription; whereas the PLC, calcium-dependent PKC and Rho kinase pathway influences CCL2 protein release. This thesis also examined the signalling receptor and downstream effectors involved in thrombin-induced CCL2 production in pHALFs. The results demonstrate that PAR_1 coupling to $G\alpha_q$ is also both necessary and sufficient in mediating thrombin-induced CCL2 production at low concentration (0.3 nM) of the proteinase, whereas at high concentrations (10 nM), these effects may be partially PAR_1-independent. This discrepancy between MLFs and pHALFs may be explained by differences of PAR receptor expression between species. Investigation of the downstream signalling pathways revealed that both ERK1/2 and Rho kinase are involved in thrombin (0.3 nM and 10 nM)-induced pHALF CCL2 release.

5.1 Signal transduction in thrombin (10 nM)-induced CCL2 production in mouse lung fibroblasts

5.1.1 Thrombin induces CCL2 production in a concentration- and time-dependent manner in MLFs

In order to delineate the signalling events that lead to CCL2 production, I first examined the effect of thrombin on MLF CCL2 production. In this study, thrombin was shown to stimulate CCL2 production in a concentration-dependent manner from 0.03 nM onwards. Strikingly, this concentration response experiment did not plateau at 300 nM, the highest concentration examined. It is possible that the maximal stimulatory concentration (300 nM) of thrombin used here is not high enough to reach a plateau. In normal human plasma, the zymogen prothrombin is present at concentrations around 1.4 μM, but concentrations of around 130 nM have been calculated to represent the maximum concentration of active thrombin generated during blood clotting *in vivo* (Walz *et al.*, 1985; Davie and Kulman, 2006). Therefore, the concentrations used in the present experiments are at near-physiological concentrations, and concentrations in excess of 300 nM are unlikely to be reached in extra vascular compartments *in vivo*. Time-course experiments showed a significantly time-dependent increase of CCL2 after thrombin (10 nM) stimulation, suggesting that thrombin-induced CCL2 protein production in MLFs is not from the pre-stored CCL2, but synthesised de novo.

5.1.2 Thrombin exerts its stimulatory effects on CCL2 release via activation of PAR_1

Thrombin exerts most of its cellular effects via activation of at least three PARs (PAR_1, PAR_3, and PAR_4) by limited proteolytic cleavage of the N-terminal extracellular domain and the unmasking of a tethered ligand (Vu *et al.*, 1991). PAR_1 is the high affinity

thrombin receptor. It is cleaved at lower thrombin concentrations and preferentially cleaved when other PARs are present. However, it is widely accepted now that thrombin can also signal via PAR_4 when relatively higher concentrations of thrombin are used, including 30 nM (Kahn *et al.*, 1999).

As both PAR_1 and PAR_4 are expressed in MLFs, and also no plateau was achieved in the concentration-response experiments in the present study, it is therefore possible that PAR_4 might be needed for thrombin responses obtained with high concentrations in MLFs. Indeed, in the present study, concentration-response experiments with PAR_1 KO MLFs showed that at 10 nM, thrombin had no effect on CCL2 production, suggesting that PAR_1 might mediate the response of thrombin at 10 nM. In contrast, significant increases in CCL2 production were observed at 30 nM and 100 nM of thrombin. This further suggests that the activation of PAR_4 might be involved in thrombin responses. However, experiments showed that the PAR_4 specific agonist peptide AYPGKF and its scrambled control peptide YAPGKF failed to induce any CCL2 production in PAR_1 KO MLFs, suggesting that PAR_4 plays no role in this response of thrombin in MLFs. As thrombin is a protease, the effects of higher concentrations may therefore be due to the release of mediators bound to the pericellular matrix. In this regard, thrombin has previously been shown to release TGF-β bound to the pericellular matrix (Taipale *et al.*, 1992).

Thrombin concentration in BALF in patients with systemic sclerosis is around 15 nM compared with about 4 nM for healthy controls (Hernandez-Rodriguez *et al.*, 1995). 10 nM thrombin, which is possibly PAR_1 specific in this study, is therefore a concentration physiologically relevant and representative of levels found in the lungs of patients with fibrotic lung disease. In addition, 10 nM is a commonly used concentration of thrombin in numerous studies (Singh *et al.*, 2007), 10 nM of thrombin was then used for all subsequent studies.

The essential role of PAR_1 in mediating CCL2 release induced by thrombin (10 nM) in MLFs was further strengthened by studies using the highly selective PAR_1 peptide agonist TFLLR. TFLLR selectively activates PAR_1 as it mimics the tethered ligand sequence of PAR_1 and directly binds to the activation site of PAR_1, bypassing the need for proteolytic cleavage of the receptor. TFLLR uniquely activates PAR_1 and unlike the other commonly used peptide SFLLR, it does not cross-activate PAR_2 in mesenchymal cells (Hollenberg *et al.*, 1997). The work presented here shows that this specific PAR_1 activating peptide TFLLR significantly induced CCL2 release in MLFs. The

concentration of TFLLR needed to stimulate this effect is usually much higher than for thrombin since PAR activating peptides are generally less efficient than the *in vivo* physiological activators of PARs (Hollenberg and Compton, 2002). This may be explained by the following possibilities. First, following cleavage of PAR_1 by thrombin, the tethered ligand may adopt a specific position that favours a more efficient activation of the receptor compared to free-floating activating peptide. Second, activation of PAR_1 by thrombin is uniquely facilitated by a hirudin-like sequence towards the N-terminus of PAR_1 which can interact with thrombin's anion-binding exosite.

It is worth mentioning that in these experiments, the CCL2 response obtained was usually greater for TFLLR over thrombin, although the concentration for TFLLR was much higher than thrombin. Similar differences in potency and efficacy between thrombin and thrombin receptor activating peptide (TRAP) have also been observed in PAR_1-mediated CCL2 release in vascular endothelial cells (Marin *et al.*, 2001). However, this may conflict with the notion that PAR activating peptides are generally less efficient for PAR activation than those *in vivo* physiological activators of PARs as described above. One possible explanation lies in the mechanism by which PAR_1 mediates the cellular response of thrombin via the activation of G-proteins, and this will be further discussed in detail in the next section (**5.1.4.1.2**)

PAR_1 KO MLFs failed to respond to TFLLR, further supporting the unique role of PAR_1 in mediating thrombin-induced CCL2 release. It should be noted that the scrambled control peptide, RLLFT at comparable concentration, did not have any effect in both MLFs and PAR_1 KO MLFs. These results support the notion that the stimulatory effects of TFLLR on CCL2 release were PAR_1-mediated, rather than the possible activation of other non-PAR receptors. In this regard, a study has demonstrated that PAR activating peptides at elevated concentrations may activate receptors other than PARs (Roy *et al.*, 1998).

Finally, the critical role of PAR_1 in thrombin-induced CCL2 release in MLFs was established by studies utilising the potent and selective PAR_1 antagonist, RWJ-58259 (Damiano *et al.*, 2003). This antagonist is a small indazole-based compound which specifically inhibits the tethered ligand of PAR_1 binding to its second extracellular loop, and thus prevents receptor activation. RWJ-58259 has been shown to significantly block PAR_1-mediating signalling events both *in vitro* and *in vivo* (Andrade-Gordon *et al.*, 2001). In the present study, RWJ-58259 inhibited thrombin-induced CCL2 release in a concentration-dependent manner from concentrations as low as 0.1 μM, with an IC_{50}

value of 0.4 μM. This antagonist completely abolished this effect of thrombin at 3 μM, a concentration that is much lower than the IC_{50} (8 μM) of RWJ-58259 previously described to block thrombin-induced platelet responses in *in vitro* culture systems (Andrade-Gordon *et al.*, 2001). Higher concentrations of RWJ-58259 are required when elevated concentrations of thrombin are present. However, thrombin concentrations in the present study were comparable with the studies by Andrade-Gordon (10 nM versus 8 nM). This higher IC_{50} of RWJ-58259 in studies by Andrade-Gordon may then be due to the potency of thrombin towards PAR_4 activation in platelets. The ability of the PAR_1 antagonist to inhibit thrombin signalling in MLFs at lower concentrations further reflects that PAR_1 is the main thrombin-sensitive receptor on these cells in the present study.

Taken together, these data provide compelling evidence that PAR_1 is the signalling receptor involved in thrombin (10 nM)-induced CCL2 release in MLFs.

5.1.3 Thrombin stimulates CCL2 mRNA levels in an immediate-early gene response fashion

In addition to the impressive induction of CCL2 at the protein level, thrombin also induced a rapid and robust increase in CCL2 mRNA levels. The increase of CCL2 mRNA levels induced by thrombin is actinomycin-D (act-D) sensitive, further suggesting that the observed increase in CCL2 release is not due to the release of pre-stored CCL2. These data are consistent with previous reports of thrombin-induced CCL2 production in endothelial cells and monocytes (Colotta *et al.*, 1994). As act-D is an inhibitor of gene transcription, the attenuation of CCL2 mRNA levels by act-D further indicates that gene transcription is likely to be an important mechanism for mediating thrombin-induced CCL2 protein release in MLFs. In addition, PAR_1 antagonism abolished thrombin-induced CCL2 mRNA levels, suggesting that PAR_1 mediates thrombin-induced CCL2 release via increased gene transcription.

CCL2 is a well-known immediate-early gene in fibroblasts that has been proposed by several studies (Cochran *et al.*, 1983; Shyy *et al.*, 1994; Sridhar *et al.*, 1999; Kinser *et al.*, 2004). Immediate-early genes are a functional diverse family of genes that are induced at transcriptional level in response to stimuli including growth factors and serum, and the induction is not dependent on new protein synthesis (Sridhar *et al.*, 1999). Indeed, results obtained in this study further confirmed this notion. The effect of thrombin on CCL2 mRNA levels in MLFs was very rapid (within 30 minutes), cycloheximide-insensitive, maximal (21 fold increase relative to control) at 6 hours, and the signal diminished after 9 hours. This suggests that the CCL2 response to thrombin

is typical of that of an immediate-early gene. For comparison, the induction of CCL2 at the mRNA level obtained with thrombin in dermal fibroblast was reported to be much delayed, prolonged and with less magnitude (Bachli *et al.*, 2003). In this study, the increase of CCL2 mRNA levels was delayed until 2.25 hours, and was prolonged up to at least 24 hours with a maximal of 4-fold increase. This is unusual for an immediate-early gene response and is not concordant with many other studies indicating that CCL2 is an immediate-early gene (Sridhar *et al.*, 1999; Kinser *et al.*, 2004). The discrepancy between the present study and work by Bachli EB and colleagues may be explained by the cell lines employed in both studies as both are fibroblasts, but derived from different organs.

It is worth mentioning that although cycloheximide did not inhibit thrombin-induced CCL2 mRNA levels in this study, cycloheximide significantly enhanced the basal and thrombin-stimulated increase of CCL2 mRNA in MLFs. Cycloheximide is an inhibitor of protein synthesis. Induction by cycloheximide is generally interpreted as the result of reduced synthesis of RNAses (Goppelt-Struebe and Stroebel, 1995). This is typical for immediate-early gene responses, the expression of which is independent of *de novo* protein synthesis. Cycloheximide-dependent increase of CCL2 mRNA levels in the present study is concordant with other studies performed in smooth muscle cells, endothelial cells and mesangial cells (Colotta *et al.*, 1992; Colotta *et al.*, 1994; Goppelt-Struebe and Stroebel, 1995).

In summary, following exposure to 10 nM thrombin, significant CCL2 protein production is obtained in MLFs. Gene transcription appears to be an important pathway for thrombin-induced CCL2 production in MLFs and *de novo* protein synthesis appears not to be required. These effects of thrombin are further mediated via PAR_1 at this concentration of the proteinase.

5.1.4 Delineation of the signalling pathways involved in PAR_1-mediated CCL2 production by mouse lung fibroblasts

Like the other important body organs, lung is a highly complex, but well-organised system in which signal transduction plays critical roles by transducing extracellular stimuli into appropriate cellular responses. Understanding the mechanisms by which extracellular stimuli modify the functions of the cells may not only provide valuable insight into the pathogenesis of lung disease, it may also lead to the identification of novel therapeutic targets for pharmacological intervention.

5.1.4.1 Role of G proteins in PAR_1-mediated CCL2 release and mRNA accumulation

5.1.4.1.1 Strategies used to study GPCR-G protein signalling

PAR_1 exerts its pluripotent cellular effects by concomitant activation of $G\alpha_{i/o}$, $G\alpha_q$ and $G\alpha_{12/13}$. These G proteins act as molecular switches that couple PAR_1 to their relevant effector systems such as kinases or ion channels. Although many of the resultant biological consequences of PAR_1 activation in fibroblasts are known, less is known about which G proteins mediate these events. Previously, unlike kinases, direct inhibitors of G proteins were rarely available with the exception of pertussis toxin for $G\alpha_{i/o}$ and this may hinder the in-depth investigation of the role of specific G protein in signal transduction studies. There are now several promising tools available which can specifically inhibit the interaction of G protein-coupled receptors with individual G proteins. In particular, the newly developed small, cell permeable molecules namely pepducins seem to be promising for thrombin signalling studies since they are specifically designed to block PAR_1-G protein coupling. Pepducins are designed to bind to the receptor-G protein interface on the inner leaflet of the plasma membrane and have been developed in the context of PAR_1 and PAR_4 signalling. However, the cross-inhibition between PAR_1 and PAR_4 by pepducins makes this type of inhibitor a potential tool for predicting an interaction between PAR_1 and PAR_4, rather than a specific inhibitor for inhibiting PAR_1-G protein interactions (reviewed in (Leger *et al.*, 2006). In addition, pepducins usually can not distinguish between different members of the same G-protein family mediating similar functional responses.

Genetic approaches, on the other hand, are a novel approach to selectively suppress or knockout the expression of distinct G-protein subunits. This approach has been extensively used in both *in vivo* and *in vitro* studies although it is not likely to be developed as a therapeutic approach for humans. The dominant-negative strategy is one of the most frequently used genetic approaches to examine the involvement of G proteins in a given physiological response mediated by GPCRs. Traditionally, the dominant-negative strategy involves generating dominant-negative constructs of the Gα subunit in which mutations are introduced at residues known to contact the magnesium ion (Slepak *et al.*, 1993; Slepak *et al.*, 1995). G proteins and small GTPase are activated after GTP binds to the α subunits following the release of GDP from the GDP-Mg^{2+} complex. The GTP is then bound very tightly as a complex with Mg^{2+}. The efficiency of this approach is therefore largely dependent on the degree to which Mg^{2+} is necessary to support GDP binding. This approach has been successfully used to generate dominant-negative constructs for small GTPase, e.g. $p21^{ras}$ (John *et al.*,

1993), as the GTPase $p21^{ras}$ forms a tight and nearly irreversible GDP-Mg^{2+} complex. However, it has been rather disappointing for making dominant-negative G proteins including $G\alpha_i$, $G\alpha_o$, $G\alpha_q$, and $G\alpha_{11}$ (reviewed in (Gilchrist *et al.*, 2002)). The possible reason for this is that the dominant-negative G proteins of Gα subunits bind Mg^{2+} in the GDP-Mg^{2+} complex with lower affinity than in the GTP-Mg^{2+} complex. In addition, these dominant-negative constructs of Gα subunits cannot usually distinguish between different members of the same G-protein family. The application of this approach is therefore quite limited.

It is now well-accepted that the carboxyl terminus of G protein α subunits are critical in G protein signalling (Reviewed in (Gilchrist *et al.*, 1998; Wess, 1998). Dominant-negative constructs targeting the carboxyl terminus can be used as competitive inhibitors of receptor-G protein interactions. In this regard, Dr Gilchrist and co-workers have generated minigene plasmid constructs that encode the 14 unique COOH-terminal sequences of various Gα subunits (Gilchrist *et al.*, 2001). Instead of competing with binding of Mg^{2+}, these G protein minigenes directly interfere with G protein-receptor interaction. The C-terminal region of various G protein α subunits has been shown to be not only the essential region for receptor contact, but also critical in determining the specificity of GPCR-G protein interactions (Hamm and Gilchrist, 1996; Hamm, 1998). This has been proposed by studies using ADP ribosylation by pertussis toxin, peptide competition, site-directed mutagenesis, peptide-specific antibodies, and chimeric proteins. For example, substituting a single amino acid has been shown to annul the ability of $G\alpha_i$ to bind the A1 adenosine receptor, further supporting the unique importance of the C-terminal region for receptor-G protein signalling (Gilchrist *et al.*, 1998). More importantly, these minigenes are able to target individual members of the Gα family. G protein minigenes are therefore very useful tools for dissecting G protein signalling pathways.

The specific $G\alpha_q$ antagonist Q94 employed in the present study was also designed according to the 11 amino acids found at the carboxyl-terminus of $G\alpha_q$. Q94 is a recently developed novel PAR_1 selective $G\alpha_q$ protein signalling antagonist (for a more detailed description and the specificity of Q94 please see Introduction Section 1.7.5.2). In the present study, G protein minigenes, the specific $G\alpha_q$ antagonist Q94 and the classical $G\alpha_{i/o}$ inhibitor pertussis toxin were therefore employed to dissect PAR_1-G proteins and downstream signal transduction in thrombin-induced CCL2 release in MLFs.

5.1.4.1.2 Thrombin exerts its stimulatory effects on CCL2 release via PAR_1 coupling to $G\alpha_q$

The G protein minigenes have been demonstrated as powerful tools for identifying the G protein that mediates a given physiological function following thrombin activation (Ellis *et al.*, 1999; Gilchrist *et al.*, 2001; Vanhauwe *et al.*, 2002). For example, thrombin-induced PAR_1 gene expression in endothelial cells is mediated by $G\alpha_i$ as this effect of thrombin can be abolished by introducing the $G\alpha_i$ minigene into the cells. This result was verified by using pertussis toxin, a well-known general $G\alpha_i$ inhibitor. In current study, the involvement of $G\alpha_i$ in PAR_1-mediated CCL2 release was also investigated by using both pertussis toxin and the $G\alpha_i$ minigene that encodes $G\alpha_i$ C-terminal sequences. Pertussis toxin catalyses the irreversible ADP-ribosylation of α subunits of the $G\alpha_{i/o}$ family, and this modification prevents the receptor-G protein interaction (Fields and Casey, 1997). The results obtained show that both pertussis toxin and the $G\alpha_i$ minigene did not interfere with PAR_1-mediated CCL2 protein release. This provides convincing evidence that $G\alpha_i$ is not necessary for PAR_1-mediated CCL2 protein release.

Interestingly, interruption of PAR_1 coupling to $G\alpha_i$ by pertussis toxin upregulated both basal and thrombin-induced CCL2 production, whereas the $G\alpha_i$ minigene had no such effect. There is one possible explanation for these discrepant results. The enhancement of basal CCL2 production suggests that baseline production is under the control of a labile repressor protein, which is not overcome by the addition of thrombin. The $G\alpha_i$ minigene specifically blocks the activation of the $G\alpha_i$ isoform only, whereas pertussis toxin blocks most of the members of the $G\alpha_{i/o}$ family which are pertussis toxin-sensitive except $G\alpha_z$. It is therefore possible that pertussis toxin blocks some members of the $G\alpha_{i/o}$ family that are responsible for regulating the labile repressor protein mentioned above.

In addition to $G\alpha_i$, PAR_1 can also interact with $G\alpha_{12/13}$ and $G\alpha_{q/11}$ family members. The role of $G\alpha_{12/13}$ and $G\alpha_q$ was also examined using $G\alpha_{12/13}$ and $G\alpha_q$ minigenes. The results demonstrated that $G\alpha_q$ but not $G\alpha_{12/13}$ is involved in PAR_1-mediated CCL2 release. It is worth mentioning that there are five members within the $G\alpha_{q/11}$ family, namely $G\alpha_{11}$, $G\alpha_{14}$, and $G\alpha_{15/16}$. In terms of the distribution of the $G\alpha_q$ family members, $G\alpha_q$ and $G\alpha_{11}$ are ubiquitously distributed across tissues, and have been detected in almost every cell type screened, including fibroblasts; whereas $G\alpha_{15/16}$ is only expressed in hematopoietic cells and the distribution of $G\alpha_{14}$ is much more limited (Shraga-Levine and Sokolovsky, 2000; Tian *et al.*, 2004; Hubbard and Hepler, 2006). It is therefore unlikely that $G\alpha_{15/16}$ and $G\alpha_{14}$ are involved in thrombin-induced CCL2

release in MLFs. As the $G\alpha_q$ minigene specifically targets $G\alpha_q$ isoform only, it is therefore likely that $G\alpha_q$ isoform participates in PAR_1-mediated CCL2 release.

By taking advantage of the newly developed PAR_1-$G\alpha_q$ antagonist Q94, the exclusive involvement of $G\alpha_q$ was then further investigated. Data from this work showed that Q94 blocked PAR_1-mediated increases in both CCL2 mRNA and protein levels in a concentration-dependent manner, which is consistent with the results obtained with $G\alpha_q$ minigene. As discussed previously, Q94 is highly specific for PAR_1-$G\alpha_q$ coupling. However, it is still important to ensure that the concentrations used here do not produce off-target effects that are independent of receptor-G protein interactions. TNF-α was chosen as the stimuli to test the specificity of Q94 as TNF-α has previously been shown to stimulate human lung fibroblast CCL2 release (Sabatini *et al.*, 2002). More importantly, TNF-α-induced signalling events do not require the interaction of receptor-G proteins since TNF-α signals via TNF receptor 1 (TNFR1) and TNFR2 which are not GPCRs (Ihnatko and Kubes, 2007). As expected, Q94 at the highest concentration did not interfere with TNF-α-induced CCL2 release, further suggesting that the inhibitory effects of Q94 on thrombin-induced CCL2 release are specific. Taken together, the data obtained with both the $G\alpha_q$ minigene and the $G\alpha_q$ antagonist Q94 strongly suggest a major role for $G\alpha_q$ in thrombin-induced CCL2 release.

It is important to point out that functional selectivity over G proteins does exist as a feature of PAR_1-G protein interaction. Functional selectivity is the ligand-dependent selectivity that can be presented when a receptor has several possible signal transduction pathways. Distinct ligands may cause different conformational change of the same receptor, which ultimately leads to the activation of separate signalling cascades of this receptor (Urban *et al.*, 2007). PAR_1 can couple to $G\alpha_{i/o}$, $G\alpha_q$ and $G\alpha_{12/13}$ family members, functional selectivity may therefore also occur in the present study. Indeed, current study has shown that the CCL2 response obtained was usually greater for TFLLR over thrombin. This can be explained by the functional selectivity of TFLLR-activated PAR_1 towards $G\alpha_q$ binding in the present study. This notion is consistent with studies showing that the peptide TFLLR-induced activation of PAR_1 alters PAR_1-G protein binding in favor of $G\alpha_q$ activation over $G\alpha_{12/13}$ (McLaughlin *et al.*, 2005). Compelling evidence has been provided that $G\alpha_q$ plays a critical role in PAR_1-mediated CCL2 response in MLFs, therefore enhanced CCL2 release obtained with TFLLR was due to the functional selectivity in $G\alpha_q$ compared to thrombin.

5.1.4.2 Role of MAP kinases in PAR_1-mediated CCL2 production

MAP kinase pathways are one of the major kinase cascades by which extracellular stimuli are transmitted as intracellular signals. There are three major members in this family, namely ERK1/2, p38s and JNKs. They have been shown to be involved in signal transduction downstream of receptor-$G\alpha_q$ coupling. In mouse lung fibroblasts, it has been clearly shown that thrombin can activate ERK1/2 and this is mediated by $G\alpha_q$ (Trejo *et al.*, 1996). However, in terms of thrombin-induced CCL2 production, several studies have suggested that the p38 kinase pathway plays a major and critical role. For example, inhibition of p38 kinase diminished thrombin-induced CCL2 production in endothelial cells (Marin *et al.*, 2001) and human smooth muscle cells (Brandes *et al.*, 2001). For other stimuli such as that obtained with the serine protease plasmin, the p38 kinase pathway is also believed to be the major signalling pathway leading to CCL2 production in human primary monocytes (Burysek *et al.*, 2002). In the present study, by using both pharmacological inhibitors and genetic approaches, ERK1/2 pathway, but not the p38 or JNK pathways, was shown to be necessary for CCL2 protein release and mRNA levels mediated by PAR_1 coupling to $G\alpha_q$.

5.1.4.2.1 Role of p38 kinase pathway

The p38 kinase pathway is strongly activated by cellular stresses, pro-inflammatory cytokines and bacterial lipopolysaccharide (Cuenda *et al.*, 1995). The involvement of this kinase in thrombin-induced CCL2 production in MLFs was examined by using the specific p38 kinase inhibitor SB 203580. In this study, the role of p38 MAPK pathway was excluded although thrombin was found to activate this pathway in a time-dependent manner in MLFs. This conclusion is based on the findings that the p38 MAPK inhibitor, SB 203580, had no effect on thrombin-induced CCL2 protein release at concentrations at which this compound effectively blocked thrombin-induced p38 phosphorylation. It is worth pointing out that in these experiments, the inactive control compound SB 202474 at the highest concentration (2 μM) used, significantly inhibited thrombin-induced CCL2 release by about 50%, suggesting that at high concentrations these compounds may exert off-target effects. It was therefore concluded that although thrombin activates p38 MAPK in MLFs, p38 is not involved in mediating PAR_1-induced CCL2 release.

As explained above, this conclusion is not in agreement with previous reports demonstrating a role for p38 MAPK in thrombin-induced CCL2 production using comparable concentrations of SB 203580 in other cell types, including human endothelial cells (Marin *et al.*, 2001) and human smooth muscle cells (Brandes *et al.*, 2001). The discrepancy between the current study in MLFs and others may have

several explanations, including important differences in both the species and the cell type examined, as well as differences in the concentrations of thrombin employed in our (10 nM is equivalent to 0.5 U/ml) and other studies (8 U/ml) (Marin *et al.*, 2001). It is generally accepted that mechanisms involved in thrombin signalling vary greatly in a concentration-dependent manner. For example, PAR_1 was reported to mediate thrombin signalling in platelets as it can be inhibited by RWJ-58259. However, cells were refractory to RWJ-58259 when thrombin was used at elevated concentrations (e.g., 30 nM), suggesting that PAR_1 is not involved in this response induced by thrombin at relatively higher concentration (Andrade-Gordon *et al.*, 1999; Andrade-Gordon *et al.*, 2001). In studies performed by Marin and colleagues, only around 40% inhibition of CCL2 release was achieved with SB203580 (2 μM), similar as the magnitude of inhibition obtained with the control peptide SB202474 in the current study. It is likely that the inhibition obtained may have been the consequence of off-target effects of SB203580, but this is not conclusive since this control peptide was not tested in parallel culture in their study. Moreover, as discussed in Section 4.1.2, thrombin is a proteinase and it is likely that at higher concentrations, CCL2 protein production may be mediated via the release of other stimulatory mediators bound to the pericellular matrix. It is therefore possible that non-PAR_1 dependent pathways lead to activation of the p38 MAPK pathway to mediate thrombin-induced CCL2 release at high concentrations of the proteinase as shown in other studies. Although concentrations around 130 nM may be generated following intra-vascular coagulation and are therefore relevant in the context of thrombin signalling in vascular cell types, we believe that thrombin concentrations in extravascular compartments and hence in the context of fibroblast signalling responses, are likely to be much lower.

5.1.4.2.2 Role of JNK pathway

Like the p38 kinase pathway, the JNK pathway is also strongly activated by cellular stresses and pro-inflammatory cytokines. However, unlike p38, there are no previous reports linking JNK to thrombin-induced CCL2 release. In the present study, the role of JNK in mediating CCL2 production induced by thrombin was excluded as inhibition of the JNK pathway with the specific JNK inhibitor, TI-JIP, did not block this effect of thrombin. Conversely, inhibition of JNK increased basal CCL2 production in a concentration-dependent manner, suggesting that JNK interferes with basal CCL2 production. Inhibition of JNK further increased thrombin-induced CCL2 production.

As already discussed for the data obtained with pertussis toxin, the enhancement in basal CCL2 production by the JNK inhibitor may also suggest that baseline production

is under the control by a labile repressor protein, which is not overcome by the addition of thrombin. Like other kinases, ERK1/2 is dephosphorylated by specific phosphatases: termed MAP kinase phosphatases (MKPs) (Sun *et al.*, 1993). MKP-1 has been shown to be greatly upregulated by thrombin in endothelial cells and plays a key role in regulating thrombin signalling (Chandrasekharan *et al.*, 2004). Therefore, in the present study, it is possible that inhibition of both the JNK pathway and $G\alpha_i$ pathway may block thrombin-induced MKP-1 activation, which in turn enhances ERK1/2 phosphorylation and CCL2 production.

5.1.4.2.3 ERK1/2 pathway is necessary and sufficient in PAR_1-mediated CCL2 production in MLFs

The ERK1/2 pathway has been reported to be essential for thrombin-mediated mitogenesis in MLFs (Trejo *et al.*, 1996). There is a wealth of evidence that ERK1/2 is important in pulmonary fibrosis as it is involved in fibroblast proliferation, differentiation, collagen production and apoptosis (Yoshida *et al.*, 2002). In the present study, by using two structurally unrelated and well documented inhibitors, U0126 and PD98259 (inhibitors of the ERK1/2 activator MEK1/2), I have been able to show that the MEK1/2 pathway is necessary for PAR_1-mediated CCL2 release. In these experiments, basal CCL2 production was not affected by the inhibition of MEK1/2, suggesting that MEK1/2 is not necessary for regulating basal CCL2 production. In contrast, inhibition of MEK1/2 blocked the production of PAR_1-mediated CCL2 production in a concentration-dependent manner from 0.3 μM onwards. As ERK1/2 is the direct downstream substrate of MEK1/2, the observations suggest that ERK1/2 is necessary for PAR_1 mediated-CCL2 production.

In order to further support the involvement of ERK1/2 in this response, the effect of PAR_1 on ERK1/2 phosphorylation induced by thrombin was then examined. Time-course data showed that ERK1/2 was rapidly phosphorylated by thrombin. This effect was transient and the signal reverted back to baseline levels within 30 minutes. Inhibition of MEK1/2 with U0126 completely abolished thrombin-induced ERK1/2 phosphorylation at 1μM. Inhibition of thrombin-induced PAR_1 activation with 1μM of the selective PAR_1 antagonist RWJ-58259 also completely abolished ERK1/2 phosphorylation. This suggests that PAR_1 is sufficient for ERK1/2 activation in mouse lung fibroblasts and is consistent with previous studies conducted by Trejo and colleagues in the context of mitogenic responses (Trejo *et al.*, 1996). The results also suggest that PAR_1 signals via ERK1/2 to induce CCL2 release.

Although U0126 and PD98259 are extensively used in both *in vivo* and *in vitro* studies, the specificity is always a concern. In the present study, MEK1 mutant constructs were employed to validate and consolidate findings from MEK1/2 pharmacological inhibitor studies. Wild type and mutant MEK1 gene constructs were introduced into MLFs in order to block or constitutively activate the ERK1/2 pathway. The wild type MEK1-pBabePuro encodes wild type rat MEK1 gene. The dominant-negative MEK1-pBabePuro construct was obtained by mutation with alanine substitution at Ser-221. The constitutively active MEK1-pBabePuro construct was derived from glutamic acid substitutions at Ser-217 and Ser-221 (Cowley *et al.*, 1994). These constructs have been successfully used to demonstrate the pivotal role of ERK1/2 in mediating fibroblast proliferation and growth-factor-induced differentiation by interfering with ERK1/2 activation in PC12 cells (Pages *et al.*, 1993; Cowley *et al.*, 1994).

In order to ensure high transfection efficiency, a retroviral transfection system was employed to deliver these constructs into MLFs. The retrovirus has a number of features that makes it unique as a gene delivery vehicle: genetic stability, high expression, and a flexible genome. Once transfected into cells, they exist as integrated proviruses in the chromatin of the host cells. The retroviral promoter can direct high-level, efficient expression of genes encoded within the confines of its genome (Zhang and Godbey, 2006). The retroviral genome can accommodate changes to its configuration, it is therefore possible for the inclusion of foreign genes. Unlike pharmacological inhibitors, this genetic approach only interferes with the kinase in question and non-specific effects can be controlled by direct comparison of results obtained with the wild type gene.

The dominant-negative MEK1 construct successfully attenuated PAR_1-mediated CCL2 production by 52±3.5% ($p<0.01$). Unlike the results obtained with U0126, dominant-negative MEK1 failed to achieve complete inhibition of this response. It is most likely that the lack of complete inhibition may be related to the transfection efficiency which was not sufficient to fully block MEK1. Indeed, only 70% knockdown of ERK1/2 phosphorylation was obtained with this approach. It is worth mentioning that although both MEK1 and MEK2 are important upstream activators of ERK1/2, MEK1 has been shown to be the major activator and 10% activation of MEK1 is sufficient to fully activate ERK1/2 (Saito *et al.*, 1994). It is therefore possible that MEK2 may compensate the effect of MEK1 in the present study, which leads to the partial ERK1/2 activation. Nevertheless, the inhibitory effect of dominant-negative MEK1 on PAR_1-mediated CCL2 production is consistent with the data obtained with the MEK1/2

inhibitor U0126. Data from both dominant-negative MEK1 and U0126 therefore strongly suggest that MEK1 and by inference ERK1/2 is necessary for PAR_1-mediated CCL2 production by MLFs. In addition, the constitutively active MEK1 mutant increased basal ERK1/2 phosphorylation. Taken together, these data suggest that MEK1 (by inference ERK1/2) is sufficient for PAR_1-mediated CCL2 production by MLFs. Moreover, inhibition of ERK1/2 phosphorylation by the PAR_1 antagonist RWJ-58259 and Q94 further supports the notion that the ERK1/2 pathway is downstream of PAR_1 coupling to $G\alpha_q$ in mediating CCL2 production in MLFs.

In summary, the data obtained using both pharmacological inhibitor and genetic approaches provide strong evidence that the ERK1/2 pathway is necessary and sufficient for PAR_1-mediated CCL2 production, whereas both JNK and the p38 pathways play no role in this response of thrombin.

5.1.4.3 Ca^{2+}-independent PKC-c-Raf -ERK1/2 pathway mediates PAR_1 activation-induced CCL2 release via increased CCL2 gene expression

The downstream signalling molecules that mediate the actions of G proteins on ERK1/2 include ras, PKC, and c-Raf kinase (Blumer and Johnson, 1994), which can activate MEK (Cobb *et al.*, 1994). It is well-established that activation of $G\alpha_q$ by PAR_1 ultimately leads to the activation of PKC (Coughlin, 2000), and c-Raf has been shown to mediate thrombin-induced ERK1/2 activation in MLFs (Trejo *et al.*, 1996). In the present work, compelling evidence has provided that both c-Raf and PKC regulated PAR_1-mediated CCL2 release via the activation of ERK1/2 in MLFs. Further evidence was provided to demonstrate that the Ca^{2+}-independent-PKC-c-Raf-ERK1/2 signalling module regulates PAR_1-mediated CCL2 release via a transcriptional mechanism.

5.1.4.3.1 The involvement of c-Raf

There are three members in the Raf kinase family: A-Raf, B-Raf and c-Raf. c-Raf is the most extensively studied in the raf gene family and is ubiquitously expressed. Classically, the c-Raf-MEK-ERK1/2 cascade is considered as a signalling module (Kolch *et al.*, 2002). Indeed, extensive in vitro evidence has demonstrated that c-Raf participates in thrombin-induced ERK1/2 activation in many cell types, including smooth muscle cells (Lin *et al.*, 2002) and fibroblasts (Trejo *et al.*, 1996). As already mentioned, the c-Raf-MEK-ERK1/2 cascade has also been shown to mediate several thrombin-induced cellular effects including mitogenesis in fibroblasts (Trejo *et al.*, 1996).

In the present study, the involvement of c-Raf in PAR_1-mediated CCL2 release was confirmed by using the specific c-Raf kinase inhibitor. This inhibitor inhibited PAR_1-mediated CCL2 release in a concentration-dependent manner. This present study also showed that c-Raf-ERK1/2 cascade is indeed a linear pathway as inhibition of c-Raf by c-Raf kinase inhibitor attenuated thrombin-induced ERK1/2 phosphorylation in a concentration-dependent manner, and total inhibition was achieved with the concentration at which PAR_1-mediated CCL2 release was also completely abolished. It is therefore highly probable that c-Raf regulates PAR_1-mediated CCL2 release via the activation of ERK1/2, in other words, the c-Raf-ERK1/2 signalling module is responsible for this response.

5.1.4.3.2 Ca^{2+}-independent PKC-c-Raf-ERK1/2 signalling module mediates thrombin-induced CCL2 release

Members of the PKC family have been shown to be involved in the regulation of cell growth/cell cycle progression and differentiation (Maruyama *et al.*, 2000; Parekh *et al.*, 2000). PKC-dependent signal transduction pathways have been found to regulate many intracellular events in fibroblasts involved in the development of fibrosis. For example, thrombin induces the differentiation of normal lung fibroblasts to a myofibroblast phenotype via the PAR_1/PKC pathway (Bogatkevich *et al.*, 2003). PKC is responsible for increased synthesis of IL-8 by lung fibroblasts and regulates thrombin-induced tenascin-C expression in systemic sclerosis (SSc) lung fibroblasts (Ludwicka *et al.*, 1992; Tourkina *et al.*, 2001). PKC is also required for $TGF\beta_1$-induced ERK1/2 activation in fibroblasts (Axmann *et al.*, 1998).

The important role of PKC in PAR_1-mediated CCL2 production in MLFs was examined using three structurally unrelated pharmacological inhibitors: Ro-318425, GF109203X, and Gö6976. PKC isozymes can be generally divided into two groups: Ca^{2+}-dependent (conventional PKC), and Ca^{2+}-independent (novel and atypical isozymes). Ro-318425 and GF109203X are regarded as broad-spectrum inhibitors that theoretically inhibit all isozymes of PKC family members, whereas Gö6976 inhibits Ca^{2+}-dependent PKC isozymes only. It was shown here that all three PKC inhibitors attenuate PAR_1-induced CCL2 production in a concentration-dependent manner. This provides strong evidence that PKC is involved in PAR_1-mediated CCL2 production in MLFs, in particular the Ca^{2+}-dependent PKC as demonstrated by Gö6976.

It has been demonstrated elsewhere that PKC is involved in thrombin-induced ERK1/2 phosphorylation. For example, in rat astrocytes, thrombin-stimulated ERK1/2 activation

has been shown to be mainly mediated by PAR_1 and this is partially mediated via the $G\alpha_q$-PKC pathway (Wang *et al.*, 2002). More importantly, activation of c-Raf-ERK1/2 cascade in MLFs by thrombin has also been reported to be mediated by PKC (Trejo *et al.*, 1996). It is therefore tempting to speculate that PKC lies in upstream of c-Raf-ERK1/2 pathway for this response of thrombin. In the present study, PKC inhibition by Ro-318425 also attenuated c-Raf and ERK1/2 phosphorylation following PAR_1 activation by thrombin. Interestingly, the Ca^{2+}-dependent PKC inhibitor Gö6976 did not inhibit this response. These data raised the possibility that the Ca^{2+}-dependent PKC and Ca^{2+}-independent PKC may be involved in PAR_1-mediated CCL2 protein production via a different mechanism. It is well-established that these PKC isozymes are not only activated via different mechanisms such as Ca^{2+}-dependent and Ca^{2+}-independent mechanisms, they also differ remarkably in mediating cellular signalling events. For example, both PKC-α and PKC-ε have been shown to mediate fibroblast collagen deposition (Fang *et al.*, 2004; Fang *et al.*, 2008). PKC-α regulates this response by inducing caveolin-1 expression, whereas PKC-ε regulates this response via the activation of ERK1/2 pathway. It is therefore not unexpected that the Ca^{2+}-dependent PKC and Ca^{2+}-independent PKC would regulate PAR_1-mediated CCL2 production via different mechanisms. This notion has been further confirmed by the effects of these PKC inhibitors on PAR_1-mediated CCL2 mRNA levels which will be discussed in the next section. To the best of my knowledge, this is the first report showing that PKC is involved in thrombin-induced CCL2 production.

5.1.4.3.3 Ca^{2+}-independent PKC-c-Raf -ERK1/2 pathway mediates PAR_1 activation induced CCL2 release via a transcriptional mechanism

The increase of CCL2 protein production induced after PAR_1 activation has been shown to be transcriptionally dependent, rather than mediated via the release of CCL2 from intra-cellular stores. As discussed in section 4.2.3.2, the linear Ca^{2+}-independent PKC-c-Raf -ERK1/2 pathway has been shown to be responsible for PAR_1-mediated CCL2 production. By using pharmacological inhibitors for these kinases, I have been able to show that inhibition of this pathway also inhibits PAR_1-induced increase of CCL2 mRNA levels. However, inhibition of Ca^{2+}-dependent PKC by Gö6976 had no effect on PAR_1-induced CCL2 mRNA accumulation. This further confirmed the notion that Ca^{2+}-dependent PKC and Ca^{2+}-independent PKC regulate PAR_1-mediated CCL2 production via different mechanisms.

5.1.4.4 The Ca^{2+}-dependent PKC-Rho kinase pathway mediates the effects of PAR_1 activation on CCL2 release via a post-transcriptional mechanism

Ca^{2+}-dependent PKC is a well-recognized downstream effector of $G\alpha_q$ and is activated in response to a rise in intracellular Ca^{2+} concentration induced by PLC activation. It is therefore possible that both PLC and Ca^{2+} may be in a linear pathway with Ca^{2+}-dependent PKC mediating PAR_1 activation-induced CCL2 production. In the present study, the data obtained show that both PLC and Ca^{2+} were involved in this process as inhibition of PLC and Ca^{2+} inhibited thrombin-induced CCL2 release in a concentration-dependent manner. Further evidence provided also indicated that PLC and Ca^{2+} may lie in the same linear pathway with Ca^{2+}-dpendent PKC to mediate this response. Like Ca^{2+}-dependent PKC, inhibition of both PLC and Ca^{2+} also had no effect on ERK1/2 phosphorylation induced by thrombin in MLFs. Furthermore, inhibition of both PLC and Ca^{2+} did not block the increase of CCL2 mRNA levels induced by PAR_1 activation, consistent with the results obtained with Ca^{2+}-dependent PKC inhibition. This led to the conclusion that PLC-Ca^{2+}-Ca^{2+}-dependent PKC lie in a linear pathway in mediating PAR_1 activation-induced CCL2 production via a non-transcriptional mechanism.

It is well-established that the activation of the α subunit of $G\alpha_q$ triggers an increase in intracellular Ca^{2+} concentration by stimulating PLC-β activity (Babich *et al.*, 1990) and that $G\alpha_{12/13}$ activation preferentially induces Rho kinase activation (Wess, 1998). Recent emerging evidence suggests that $G\alpha_q$ could be another major G protein to signal via RhoA/Rho kinase, although how this occurs at the molecular level is not well understood (Booden *et al.*, 2002; Chikumi *et al.*, 2002; Vogt *et al.*, 2003). However, $G\alpha_q$-dependent RhoA activation appears to be dependent on the receptors being stimulated and the cell types under investigation as there is also evidence against the primary role of $G\alpha_q$ in receptor-mediated RhoA activation (Reviewed in (Sah *et al.*, 2000)). Despite the controversy, PAR_1 is one of the receptors that have been shown to activate RhoA via coupling to $G\alpha_q$ (Vogt *et al.*, 2003; Singh *et al.*, 2007). In particular, a recent study by Singh and colleagues demonstrated that $G\alpha_q$ can lead to Rho kinase activation via PLCβ-Ca^{2+}-dependent PKC to mediate thrombin-induced endothelial cell contraction (Singh *et al.*, 2007). RhoA is one the best characterized members of the small Rho GTPase family and Rho kinase is a well-known major target of RhoA (Kaibuchi *et al.*, 1999). RhoA/Rho kinase has been shown to be involved in many cellular responses triggered by thrombin, such as smooth muscle contraction, cell migration, fibroblast proliferation and transformation (reviewed in (Sah *et al.*, 2000)). Specifically, thrombin-induced lung fibroblast proliferation has been shown to be mediated by the PKC-RhoA pathway (Bogatkevich *et al.*, 2003). The present study has

demonstrated that the PLC-Ca^{2+}-dependent PKC pathway mediate PAR_1 activation-induced CCL2 release independent of ERK1/2 activation. It was therefore possible that this pathway acts through RhoA/Rho kinase instead.

C3 exoenzyme is the RhoA inhibitor that irreversibly ADP-ribosylates RhoA. This inhibitor blocked thrombin-induced CCL2 production, suggesting that RhoA is involved. It is worth mentioning that C3 exoenzyme did not fully block this response because only relatively low concentrations of C3 exoenzyme were employed. At elevated concentrations, C3 exoenzyme caused cells to die. Inhibition of Rho kinase using two structurally unrelated inhibitors Y-27632 and H-1152 also blocked this response. Taken together, these data support the notion that RhoA/Rho kinase is involved in PAR_1 activation-induced CCL2 release. However, RhoA/Rho kinase did not mediate thrombin-induced ERK1/2 phosphorylation or CCL2 mRNA accumulation since inhibition of RhoA/Rho kinase by C3 exoenzyme and Y-27632 had no effect on these responses. This is consistent with the finding that PLC-Ca^{2+}-dependent PKC pathway also mediates the effects of thrombin via an ERK1/2-independent and post-transcriptional pathway. This raised the possibility that RhoA/Rho kinase is downstream of PLC-Ca^{2+}-dependent PKC pathway and mediates thrombin-induced CCL2 release.

In the present study, further compelling evidence was provided that RhoA/Rho kinase activation is mediated by a $G\alpha_q$-PLC-Ca^{2+}dependent PKC pathway to mediate thrombin-induced CCL2 release. First, myosin light chain (MLC) was demonstrated to be involved in thrombin-induced CCL2 release via a post-transcriptional mechanism as inhibition of MLC by blebbistatin inhibited thrombin-induced CCL2 release, but had no effect on CCL2 mRNA accumulation. MLC is one of the major downstream substrates of RhoA/Rho kinase (Amano *et al.*, 1996). Rho kinase can activate MLC directly via phosphorylation or indirectly by phosphorylating the myosin-binding subunit of MLC phosphatase, which in turn renders the phosphatase inactive and thus prevents MLC dephophorylation (Kimura *et al.*, 1996). Second, in the present study, inhibitors of PLC, Ca^{2+} and Ca^{2+}-dependent PKC, RhoA and Rho kinase all blocked thrombin-induced phosphorylation of MLC. Third, a direct link between this pathway and $G\alpha_q$ coupling to PAR_1 was demonstrated by showing that the novel PAR_1 selective $G\alpha_q$ antagonist (Q94) also abolished thrombin-induced MLC phosphorylation. Taken together, these data led us to conclude that the PLC-Rho kinase pathway mediates PAR_1-activation-induced CCL2 release in MLFs via a post-transcriptional mechanism.

Although Rho kinase was first characterized as a major regulator of actin dynamics, it is now well recognised that Rho family proteins influence a range of other cellular processes, including gene transcription and protein secretion (reviewed in (Etienne-Manneville and Hall, 2002)). In the present study, we provide evidence that the Rho kinase pathway is involved in PAR_1 mediated CCL2 protein release post-transcriptionally, likely by influencing protein secretion. This is based on two observations. First, inhibition of Rho kinase only blocked thrombin-induced CCL2 protein release but not CCL2 mRNA accumulation. Second, immunocytofluoresence experiments to elucidate the mechanism by which the Rho kinase pathway influences CCL2 release, showed that intracellular protein production was blocked with the MEK1/2 inhibitor, U0126, but was unaffected by the Rho kinase inhibitor, Y27632. Taken together, these data indicate that blockade of Rho kinase signalling is not essential for PAR_1-mediated CCL2 gene expression and subsequent intracellular protein production. Given that Y-27632 does block PAR_1-mediated increases in CCL2 protein levels measured in cell culture supernatants, this thesis proposes that this pathway most likely affects CCL2 protein secretion.

5.1.4.5 Speculation of the involvement of RhoGEFs in RhoA activation by $G\alpha_q$

It is worth noting that although $G\alpha_q$ can activate RhoA/Rho kinase to mediate downstream cellular events, as suggested by the current study and others; whether the Rho GEFs are required for RhoA activation by $G\alpha_q$ is still a controversial issue. It is well established that the activation of RhoA requires its dissociation from the GDP/GDIs (Rho GDP dissociation inhibitors) complex followed by GTP exchange mediated by RhoGEFs. In terms of RhoA activation by GPCRs, the RhoGEF proteins, p115RhoGEF, PDZ-RhoGEF, p63RhoGEF and Leukemia-associated Rho guanine-nucleotide exchange factor (LARG) have been shown to stimulate Rho kinase activity (Hart *et al.*, 1998; Kozasa *et al.*, 1998; Fukuhara *et al.*, 2001; Suzuki *et al.*, 2003; Lutz *et al.*, 2007). In particular, p63RhoGEF has been shown to be a downstream effector of $G\alpha_q$ and interact selectively with $G\alpha_{q/11}$, but not $G\alpha_{12/13}$ (Lutz *et al.*, 2005; Lutz *et al.*, 2007). However, p63RhoGEF binding to $G\alpha_q$ has been shown to occur competitively with PLC-β, and inhibition of both PLC-β and PKC do not effect p63RhoGEF-$G\alpha_q$ activation of RhoA (Lutz *et al.*, 2005). These findings provide compelling evidence that p63RhoGEF is a downstream effector of $G\alpha_q$ that is independent of PLC-β. In contrast, current study proposed that PLC-β mediated $G\alpha_q$-induced RhoA activation as inhibition of PLC-β inhibited MLC phosphorylation. It is therefore unlikely that p63RhoGEF participates in $G\alpha_q$-RhoA activation in terms of cellular responses examined in this study.

Moreover, the findings presented here and those reported by Singh and colleagues contrast with another previous report demonstrating that $G\alpha_q$-mediated Rho kinase activation occurs independently of PLC-β in mouse embryonic fibroblasts (MEFs) (Vogt *et al.*, 2003). Singh and colleagues provide evidence that $G\alpha_q$-mediated Rho kinase activation is p115RhoGEF dependent following PLC-Ca^{2+}-dependent PKC activation; whereas in studies by Vogt and colleagues, thrombin-induced Rho kinase activation in MEFs was shown to be mediated by the LARG. These differing observations may have several explanations. First, they may be related to cell type. From my experience, I am aware that thrombin is unable to induce CCL2 production in MEFs so that thrombin responses are cell-type specific. Second, they may also be explained by differences in the concentrations of thrombin employed. The current work and studies by Singh and colleagues used 10 nM thrombin (10 nM is equivalent to 0.5 U/ml), whereas studies by Vogt and colleagues in MEFs employed 1 U/ml thrombin. As discussed before, mechanisms leading to a given signalling event of thrombin are concentration-dependent so that the comparison of responses at different concentrations may not be easily comparable. Taken together, the findings mentioned here are consistent with a PLC-Ca^{2+}-dependent PKC-dependent Rho kinase activation mechanism rather than a LARG-mediated mechanism. It is therefore tempting to speculate that PAR_1-$G\alpha_q$ may also signal via p115RhoGEF to activate Rho kinase for thrombin-induced CCL2 release in MLFs, but this would require confirmation.

5.1.5 Specificity of the inhibitors used

Each protein kinase phosphorylates more than one protein, and one protein phosphatase also dephosphorylates more than one protein. Therefore, inhibiting most protein kinases and phosphatases will alter the phosphorylation of many proteins, perhaps giving rise to unwanted side effects (Cohen, 1999). It is unlikely that small cell-permeable molecules could be developed to inhibit one kinase specifically without inhibiting at least a few others. Even if a drug inhibits just one out of several protein kinases tested, there is no guarantee that it does not inhibit other protein kinases or other types of enzymes that have not been examined (Davies *et al.*, 2000). In cell signalling studies, it is important to minimize the risk of off-target effects. In order to address the concerns regarding the specificity of inhibitors, I designed my experiments according to the recommendations from previous studies (Davies *et al.*, 2000; Bain *et al.*, 2003; Bain *et al.*, 2007) and our own experience. However, confirmation of the selectivity of PAR_1 antagonists and G proteins inhibitors used in current study needs further clarification.

First, I selected potent and cell-permeable protein kinase inhibitors whose specificities have been tested towards a wide range of protein kinases *in vitro* (Davies *et al.*, 2000; Bain *et al.*, 2003; Bain *et al.*, 2007). For example, it is well-recognised that SB 203580 (p38 kinase inhibitor) and U0126 (MEK1/2 inhibitor) have the most impressive selectivity profiles (Davies *et al.*, 2000). In particular, U0126 is not an ATP-competitive inhibitor but acts by suppressing the activation of MEK1, whereas the inhibitors that are ATP-competitive are usually less selective and require higher concentration to suppress the phosphorylation of a substrate in cells. TI-JIP has been recognized as a unique and most potent inhibitor of JNK activity when compared with other compounds such as sp600125 (Bain *et al.*, 2003; Borsello *et al.*, 2003; Bain *et al.*, 2007). The PKC inhibitor Ro-318425 is also a potent inhibitor based on Ro-318220 that has been tested by others (Davies *et al.*, 2000). Y-27632 is a typical Rho kinase inhibitor that has been extensively used by investigators.

Second, the role of the targeting kinases was verified by more than one structurally-unrelated inhibitor. For instance, the involvement of ERK1/2 was confirmed by both U0126 and PD98059. Similarly for PKC, I employed the broad-spectrum PKC inhibitors, Ro-318425 and GF 109203X, and the Ca^{2+}-specific PKC inhibitor Gö6976. In addition, for Rho kinase, two compounds were used: Y-27632 and H-1152.

Third, wherever available, inactive control compounds for each inhibitor were tested in parallel cultures. For example, SB202474 was used as a negative control for p38 kinase inhibitor SB203580, and U73343 was used as a negative control for PLC kinase inhibitor U73122. This was particularly important when high concentrations of the active compounds were used.

Fourth, for each inhibitor, a full range of concentrations was tested in order to determine the specific IC_{50}. The IC_{50} of an inhibitor varies between cell-based and non cell-based assays; it is also determined by cell type and different stimuli. For instance, the concentration of U0126 that suppresses the activation of MEK1 by 50% in Swiss 3T3 cells is 70 nM, whereas the concentration required to inhibit MEK1 activity by 50% in kinase assays was 13 μM which is 200-fold higher (Davies *et al.*, 2000). In the current study, I employed concentrations of U0126 which provided no inhibition (0.03 μM) to almost 100% (10 μM) inhibition of CCL2 production in MLFs, the final IC_{50} of U0126 in this study was determined to be 1.3 μM. Similarly, the IC_{50} of Ro-318425 is 15 nM for rat brain PKC obtained in non cell-based protein assay (Wilkinson *et al.*, 1993), whereas the IC_{50} in the present study was determined to be 0.93 μM.

Fifth, in order to further validate and consolidate the results obtained with pharmacological inhibitors, the role of the critical components in the signalling pathways was further assessed using a genetic approach. For example, although the role of ERK1/2 was examined using the well-recognized MEK1/2 inhibitors U0126 and PD98059, genetic MEK1 mutants were employed to strengthen the results obtained. In addition, the critical involvement of $G\alpha_q$ was also established based on the results obtained with both PAR_1 specific $G\alpha_q$ antagonist and $G\alpha_q$ minigene approaches.

Finally, I demonstrated that most inhibitors blocked CCL2 release at the same concentrations that prevented the phosphorylation of an authentic physiological substrate of the protein kinase. For example, ERK1/2, p38 MAPK and MLC phosphorylation was assessed to identify the lowest concentration of respective inhibitors required for subsequent CCL2 release experiments. The concentration of each inhibitor used for subsequent CCL2 release experiments was also determined based on the IC_{50} value obtained in these experiments. In addition, the basal effect of each inhibitor was also monitored in parallel in order to minimize off-target or non-specific toxic effects.

5.1.6 Integration of the flow of information

The recent explosion of knowledge on the basic mechanisms regulating signal transmission now affords the unique opportunity to begin unravelling the intricacies of how these signals are integrated in space and time to elicit a final biological response. In this study, we provide evidence that thrombin-induced CCL2 release is mediated via coupling of PAR_1 to $G\alpha_q$ and the cooperation between ERK1/2 and Rho kinase signalling pathways. This raises the question as to how the flow of information to the two pathways, one directed at the nucleus the other at a specific secretory apparatus, is regulated? Signal transduction leading to cellular responses is a complex processes initiated by protein-protein interactions between ligands, receptors and kinases. Recent hypotheses including the formation of lipid rafts and 'transduceosomes' have shed light on how these multiple components may work in harmony.

Lipid rafts are specialized structures on the plasma membrane that have an altered lipid composition as well as links to the cytoskeleton. Recent studies indicate that some GPCRs, G-proteins, and their effectors localize to lipid rafts or dynamically move in and out of microdomains (Simons and Toomre, 2000). A recent study has shown that PAR_1 is present in endothelial cell plasma membrane rafts and caveolae, and that the localization of PAR_1 specifically to rafts serves as an important mechanism for the

regulation of thrombin-induced cytoskeletal changes in endothelial cells. Of particular interest to the current work, these authors further demonstrate a role for lipid rafts in mediating PAR_1 activation of both RhoA/Rho kinase signalling and MLC phosphorylation (Carlile-Klusacek and Rizzo, 2007). Since PAR_1 coupling to $G\alpha_q$ mediates thrombin-induced CCL2 secretion via the RhoA/Rho kinase pathway, we propose that it is likely that the components for secretion in this pathway might integrate on lipid rafts, but this clearly requires confirmation. In contrast, the role of lipid rafts in MAPK signalling remains controversial. For example, $G\alpha_{q/11}$ has been shown to activate p38 MAPK via lipid rafts, whereas, two studies have shown that $G\alpha_q$ does not mediate ERK1/2 phosphorylation via such rafts (Hiol *et al.*, 2003; Sugawara *et al.*, 2007). In this study, we report that PAR_1 coupling to $G\alpha_q$ mediates thrombin-induced CCL2 production via ERK1/2 rather than p38 signalling so that current evidence would lead us to propose that the components involved in the transcriptional response may not float together on lipid rafts and would therefore be segregated from the components involved in CCL2 secretion.

It is also possible that these pathways involve the assembly of a 'transduceosome' which would act as a relay to assemble and integrate the signals derived from the two pathways in order to optimize the amplitude of downstream signalling. Transduceosomes are most well described in terms of the essential role of the scaffold and anchoring protein, A-kinase anchor proteins (AKAP) in cAMP signalling (Feliciello *et al.*, 2001). cAMP-dependent protein kinase is targeted to discrete subcellular locations by the AKAPs. Localization recruits protein kinase A (PKA) holoenzyme close to its substrate/effector proteins, directing and amplifying the biological effects of cAMP signalling. Although AKAPs were identified on the basis of their interaction with PKA, AKAPs bind other signalling molecules, principally phosphatases and kinases, including notably PKC (Feliciello *et al.*, 2001). It is therefore tempting to speculate and propose a role for both lipid rafts and tansduceosomes to integrate and assemble the signalling components leading from PAR_1 activation of $G\alpha_q$ to CCL2 gene transcription via the ERK1/2 pathway and CCL2 release via the RhoA/Rho kinase pathway.

5.1.7 Summary

This work presented here provides compelling evidence that thrombin mediates its potent stimulatory effects on MLF CCL2 production and release by PAR_1 coupling to $G\alpha_q$ and the cooperation between ERK1/2 and Rho kinase signalling pathways (Figure 5.1). More specifically, the experiments conducted in chapter 3 have shown that:

(i) Thrombin stimulates MLF CCL2 protein production and upregulates its mRNA levels, suggesting that thrombin induces CCL2 production via a transcriptional mechanism.

(ii) The stimulatory effects of thrombin on MLF CCL2 release and mRNA levels are mediated by PAR_1 coupling to $G\alpha_q$ as inhibition of PAR_1-$G\alpha_q$ interaction by the novel PAR_1-$G\alpha_q$ antagonist Q94 inhibited thrombin-induced CCL2 release and mRNA levels.

(iii) Ca^{2+}-independent PKC-c-Raf-ERK1/2 pathway mediates thrombin-induced CCL2 release by influencing CCL2 gene transcription as inhibition of this pathway not only blocked thrombin-induced CCL2 release measured with the cultured supernatant, but also inhibited intracellular CCL2 protein production and CCL2 mRNA levels.

(iv) In contrast, the PLC/Ca^{2+}/Ca^{2+}-dependent PKC/Rho kinase pathway is involved in thrombin-induced CCL2 release by post-transcriptional mechanism, possibly via protein secretion. This notion is based on the observations that inhibition of this pathway attenuated thrombin-induced CCL2 release but not CCL2 mRNA levels. The possible involvement of this pathway in CCL2 protein secretion has been further demonstrated by experiments showing that inhibition of Rho kinase by Y-27632 failed to block thrombin-induced intracellular CCL2 protein accumulation measured by immunofluoresecent.

(v) Blockade of PAR_1 coupling to $G\alpha_q$ was found to inhibit both ERK1/2 and MLC phosphorylation induced by thrombin, indicating that these two pathways lie downstream of PAR_1 coupling to $G\alpha_q$ in mediating the stimulatory effects of thrombin on MLFs.

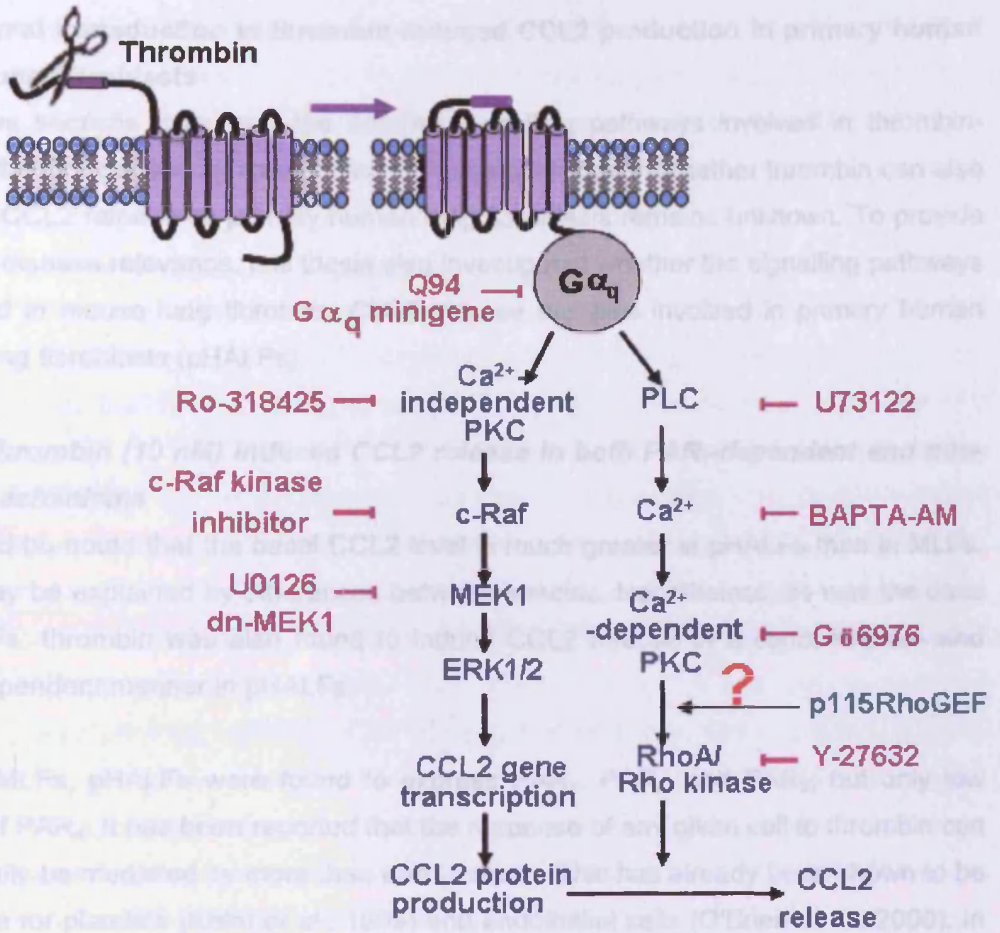


Figure 5.1 Proposed mechanisms for the signalling pathways involved in CCL2 release induced via the activation of PAR_1 by thrombin in mouse lung fibroblasts. Thrombin ligation of PAR_1 stimulates $G\alpha_q$. Activation of Ca^{2+}-independent PKC leads to the sequential activation of c-Raf, MEK1 and ERK1/2 to stimulate CCL2 gene transcription. $G\alpha_q$ also leads to the activation of PLC, Ca^{2+}, Ca^{2+}-dependent PKC to activate Rho kinase (likely via p115RhoGEF, (Singh *et al.*, 2007)) to mediate CCL2 protein release.

5.2 Signal transduction in thrombin-induced CCL2 production in primary human adult lung fibroblasts

Previous sections discussed the detailed signalling pathways involved in thrombin-induced CCL2 release by mouse lung fibroblasts. However, whether thrombin can also induce CCL2 release by primary human lung fibroblasts remains unknown. To provide human disease relevance, this thesis also investigated whether the signalling pathways involved in mouse lung fibroblast CCL2 release are also involved in primary human adult lung fibroblasts (pHALFs).

5.2.1 Thrombin (10 nM) induces CCL2 release in both PAR_1-dependent and non-PAR mechanisms

It should be noted that the basal CCL2 level is much greater in pHALFs than in MLFs. This may be explained by differences between species. Nonetheless, as was the case for MLFs, thrombin was also found to induce CCL2 release in a concentration- and time-dependent manner in pHALFs.

Unlike MLFs, pHALFs were found to express PAR_1, PAR_2, and PAR_3, but only low levels of PAR_4. It has been reported that the response of any given cell to thrombin can potentially be mediated by more than one receptor. This has already been shown to be the case for platelets (Kahn *et al.*, 1999) and endothelial cells (O'Brien *et al.*, 2000). In the present studies with pHALFs, the possibility that more than one receptor is involved in this response to thrombin at 10 nM was examined.

5.2.1.1 Thrombin (10 nM)-induced pHALF CCL2 release is partially PAR_1-$G\alpha_q$-dependent

Previous experiments with MLFs showed that PAR_1 is necessary and sufficient in mediating thrombin-induced MLF CCL2 release at 10 nM of this proteinase. Further experiments demonstrated that PAR_1 is also important in pHALF CCL2 release. First, the PAR_1 specific agonist peptide, TFLLR, was found to induce pHALF CCL2 release in a concentration-dependent manner and this response could be inhibited with PAR_1 specific antagonist RWJ-58259, suggesting that PAR_1 is sufficient for mediating pHALF CCL2 release. Second, although FXa is also an activator of PAR_2, it is well-accepted that FXa signals mainly via PAR_1 in human fibroblasts (Blanc-Brude *et al.*, 2005). FXa-induced pHALF CCL2 release was indeed inhibited by RWJ-58259, further suggesting the involvement of PAR_1 in mediating pHALF CCL2 release. Third, by taking advantage of two previously developed monoclonal antibodies with defined epitopes within the PAR_1 NH_2 terminus that prevent the cleavage of PAR_1 by thrombin, work presented

here shows that PAR_1 is necessary for mediating thrombin-induced pHALF CCL2 release as both antibodies (ATAP2 and WEDE15) significantly blocked this response of thrombin. Fourth, the $G\alpha_q$ selective PAR_1 antagonist Q94 blocked this effect of thrombin, further suggesting that PAR_1 is important, and moreover that PAR_1 coupling to $G\alpha_q$ is involved in mediating thrombin-induced pHALF CCL2 release.

However in pHALFs, the PAR_1 specific antagonist RWJ-58259 failed to inhibit this response of thrombin (10 nM). In contrast, RWJ-58259 completely abolished low concentration (0.3 nM) thrombin-induced CCL2 release. These data are consistent with previous reports showing that in human platelets, complete antagonism of thrombin with RWJ-58259 can be achieved at low thrombin concentrations, but the effect diminishes as thrombin levels rise above 10 nM (Andrade-Gordon *et al.*, 2001). There are several potential explanations. First, this could have been due to insufficient inhibition of PAR_1 after thrombin stimulation by RWJ-58259. The highest concentration of RWJ-58259 (1 μM) used for pHALFs was lower than that (3 μM) used in MLFs as this antagonist affected baseline CCL2 at high concentrations. Second, RWJ-58259 is a competitive antagonist and the effectiveness of this antagonist is dependent on the degree of binding site occupancy by the tethered ligand after thrombin cleavage. Thrombin used in these studies was of human plasma origin and it is possible that the efficacy by which thrombin cleaves PAR_1 in pHALFs is greater than in MLFs. Third, RWJ-58259 is a reversible inhibitor and the off-rate may be faster for human PAR_1 than murine PAR_1. Fourth, transactivation of PAR_2 by cleaved PAR_1 may occur when RWJ-58259 is present in pHALFs. RWJ-58259 inhibits PAR_1 activation by competing with the tethered ligand, but does not prevent unmasking of the tethered ligand. The unmasked tethered ligand of PAR_1 may therefore in turn activate PAR_2 that is located sufficiently close to PAR_1. In this regard, studies by O'Brien and colleagues have demonstrated that PAR_1 can transactivate PAR_2 to mediate thrombin-induced endothelial cell responses when PAR_1 activation is blocked (O'Brien *et al.*, 2000). In addition, PAR_2 agonist peptide was found to induce pHALF CCL2 release, further suggesting that PAR_2 is sufficient to mediate CCL2 release in these cells.

It is worth mentioning that full blockade of thrombin-induced pHALF CCL2 release at 10 nM was not obtained when PAR_1 cleavage was inhibited using PAR_1 antibodies. This may be explained by the incomplete inhibition of PAR_1 cleavage by thrombin in the presence of antibodies. However, pre-incubation time and concentration of both antibodies were chosen according to previous studies performed by O'Brien and colleagues, where the complete inhibition of PAR_1 cleavage was obtained with the

combination of ATAP2 and WEDE15, even at high thrombin concentration (50 nM) over prolonged periods of time (O'Brien *et al.*, 2000). In addition, the $G\alpha_q$ selective PAR_1 antagonist Q94 also only partially inhibited this response of thrombin in pHALFs. The maximal inhibition achieved was 53% and the effect of Q94 was saturated at 3 μM. Unlike RWJ-58259, Q94 is an allosteric antagonist that is uncompetitive. The effect of Q94 is not dependent on the degree of binding site occupancy by the tethered ligand after thrombin cleavage. Incomplete inhibition obtained with Q94 therefore suggests that PAR_1 coupling to $G\alpha_q$ is only partially involved in thrombin-induced pHALF CCL2 release at high concentrations (≥ 10 nM) of the proteinase.

Taken together, these data indicate that thrombin at 10 nM induces pHALF CCL2 release via both PAR_1-dependent and PAR_1-independent mechanisms. It is well-established that thrombin can signal via more than one receptor when multiple receptors are expressed, it is therefore possible that other PARs or non-PAR mechanisms are involved in mediating this response. These potential mechanisms will now be discussed in further detail.

5.2.1.2 Thrombin (10 nM)-induced pHALF CCL2 release is partially mediated via PAR-independent mechanism

As already mentioned, PAR_2 is not a thrombin receptor, but emerging evidence suggests that PAR_2 can be transactivated by PAR_1 and therefore contribute to the cellular responses of thrombin, particularly in human endothelial cells (O'Brien *et al.*, 2000; Lidington *et al.*, 2005). These studies propose that cleaved PAR_1 may donate its tethered ligand to PAR_2 that is located sufficiently close to PAR_1, especially when the PAR_1 antagonist is present. In current study, although RWJ-58259 did not inhibit this response of thrombin at 10 nM and the PAR_2 agonist peptide SLIGKV induced pHALF CCL2 release, it is unlikely that thrombin-induced PAR_1-independent CCL2 release is mediated by PAR_2. First, the PAR_1 specific agonist TFLLR was found to induce pHALF CCL2 release in a concentration-dependent manner and this effect could be completely blocked with RWJ-58259. Second, PAR_1 cleavage is required for PAR_2 transactivation. At 10 nM, thrombin is still able to induce pHALF CCL2 release after inhibition of PAR_1 cleavage by both ATAP2 and WEDE15. Most importantly, the $G\alpha_q$ selective PAR_1 antagonist Q94 which does not interfere with PAR_1 cleavage inhibited this response of thrombin. As discussed before, it is likely that PAR_2 plays a role in this response of thrombin at 10 nM only when PAR_1 activation is blocked by RWJ-58259.

In addition to PAR_1, PAR_4 is another important functional receptor for thrombin. In human platelets, PAR_1 and PAR_4 mediate aggregation and secretion in response to thrombin, whereas in murine platelets, thrombin triggers signalling through PAR_3 and PAR_4 (Nakanishi-Matsui *et al.*, 2000). In the present study, PAR_4 is expressed at very low levels compared with the other PARs, and the PAR_4 agonist, AYPGKF at a comparable concentration (200 μM) used in studies by others (Bilodeau and Hamm, 2007) failed to induce CCL2 release in pHALFs. These data suggest that PAR_4 is not involved in thrombin-induced CCL2 release.

Although PAR_3 is also a thrombin receptor, the ability of PAR_3 to generate intracellular signals remains doubtful since it lacks the cytoplasmic tail domain which couples to G proteins in other PARs (Ishihara *et al.*, 1997). However, emerging evidence suggests that PAR_3 not only acts as a co-factor for PAR_4 (Kahn *et al.*, 1998), but also functions as an allosteric regulator of PAR_1 signalling (McLaughlin *et al.*, 2007). More recently, a study reported that PAR_3 may signal to mediate thrombin-induced IL-8 release in A549 cells (Ostrowska and Reiser, 2008). Despite this evidence, whether PAR_3 can generate intracellular signals in fibroblasts remains unknown. In the current studies, the role of PAR_3 in thrombin-induced CCL2 release was not examined. Ongoing studies in the host laboratory have shown that PAR_1 is the principal receptor involved in thrombin-induced CCL2 release in A549 cells (Mercer et al, in revision). However, whether PAR_3 plays a role in mediating this response of thrombin (10 nM) in pHALFs remains at present an unsolved issue. Although the current evidence would suggest that this is unlikely, future studies with PAR_3 siRNA may shed light on this issue but were beyond the scope of the present work.

Taken together, current evidence suggests that thrombin at concentrations of 10 nM induces CCL2 release in pHALFs via both PAR_1-dependent and PAR-independent mechanisms. Although the data presented do not allow us to draw any fine conclusion, there is a slight possibility that the PAR-independent mechanism may involve the release of CCL2 bound to the pericellular matrix by thrombin at 10 nM.

5.2.2 PAR_1 coupling to $G\alpha_q$ plays a major role in mediating low concentration (0.3 nM) thrombin-induced pHALF CCL2 release

Previous studies have demonstrated that thrombin at different concentrations mediates the cellular responses via different mechanisms (reviewed in (O'Brien *et al.*, 2001)). Indeed, findings from pHALFs in the present study are consistent with this notion. The data obtained led to the conclusion that PAR_1 is the major receptor involved in

mediating thrombin-induced pHALF CCL2 release at 0.3 nM as the PAR_1 specific antagonist, RWJ-58259, and anti-PAR_1 antibodies completely abolished this response of thrombin. Moreover, the $G\alpha_q$ selective PAR_1 antagonist Q94 also completely blocked this response, further suggesting that PAR_1 coupling to $G\alpha_q$ plays a critical role in low concentration thrombin-induced CCL2 release in pHALFs. As discussed previously, thrombin used in this thesis may be more efficient in human cells as it is derived from human plasma. The data therefore also suggest that low concentration thrombin used in pHALFs may function via the same mechanism as shown in MLFs with 10 nM thrombin.

5.2.3 ERK1/2 and Rho kinase are involved in thrombin-induced pHALF CCL2 release

In MLFs, thrombin was found to induce CCL2 production and release via PAR_1 coupling to $G\alpha_q$ and the cooperation between ERK1/2 and Rho kinase signalling pathways. In primary human lung fibroblasts, inhibition of ERK1/2 and Rho kinase also inhibited this response of thrombin at both high and low concentrations of thrombin. These data suggest that despite the differences observed for the involvement of PAR_1 in both murine and human cells, thrombin signals via similar downstream pathways to mediate CCL2 release in murine and human lung fibroblasts.

In summary, the work performed with pHALFs provides compelling evidence that thrombin at high concentration (10 nM) mediates pHALF CCL2 release via PAR_1 coupling to $G\alpha_q$ and PAR-independent mechanisms, whereas low concentration (0.3 nM) thrombin mediates these effects mainly via PAR_1 coupling to $G\alpha_q$. In addition, downstream ERK1/2 and Rho kinase signalling play important roles in mediating thrombin-induced pHALF CCL2 release at both high and low concentrations of thrombin.

5.3 Summary and conclusion

This thesis examined the hypothesis that PAR_1 activation leads to the production and release of the pro-inflammatory and pro-fibrotic chemokine, CCL2, via its interaction with heterotrimeric G-proteins and activation of multiple downstream signalling cascades. The work performed with both MLFs and pHALFs provides compelling evidence that PAR_1 coupling to $G\alpha_q$, and downstream ERK1/2 and Rho kinase signalling are involved in thrombin-induced CCL2 release. However, data also suggest that 10 nM thrombin induces pHALF CCL2 release partially via PAR-independent mechanism, which contrasts with findings in MLFs where 10 nM thrombin induces

CCL2 release mainly via PAR_1. The differences in receptor utilization between MLFs and pHALFs may be explained by differences of PAR receptor expression between species. In addition, current studies also show that low concentration (0.3 nM) thrombin mediates pHALF CCL2 release via the same mechanism as shown in MLFs with 10 nM thrombin.

In conclusion, this thesis represents the first demonstration of the complex cooperation between ERK1/2 and Rho kinase pathways in mediating the stimulatory effects of thrombin, or indeed any other extracellular stimulus, on the induction and release of the potent chemoattractant, CCL2.

5.4 Therapeutic implications

Current therapeutic intervention for fibroproliferative pulmonary diseases is inadequate and rather disappointing. The lung offers a rich opportunity for the development of therapeutic strategies as it interfaces with both air and blood, and drugs can be directly delivered to the lung by aerosol as well as oral and parenteral routes. However, the lung also poses a multifaceted dilemma since it contains both a pulmonary and systemic circulation and consists of numerous cell types. In addition, in the pathogenesis of pulmonary fibrosis, the number of inducers, cell types, secondary mediators, chemical changes, immune responses and tissue modifications are overwhelming. This further adds difficulties to the development of therapeutic strategies targeting this complexity. Detailed studies on signal transduction through which extend stimuli induce relative cellular responses may therefore allow the identification of novel therapeutic targets.

5.4.1 Therapeutic implications of PAR_1 in pulmonary fibrosis

In the context of fibrotic lung disease, PAR_1 is emerging as an important receptor. This thesis and previous work from the host laboratory has demonstrated a major role for PAR_1 in mediating the cellular effects of thrombin on fibroblasts, including fibroblast proliferation, differentiation, collagen production and cytokine release (Chambers *et al.*, 1998; Blanc-Brude *et al.*, 2005; Howell *et al.*, 2005); Krupiczojc, unpublished; Mercer, in revision). *In vivo* studies in the bleomycin model of pulmonary fibrosis have shown that PAR_1 deficiency is protective and associated with a reduction in lung oedema, inflammatory cell recruitment and fibrosis (Howell *et al.*, 2005; Jenkins *et al.*, 2006). This points to a central role for PAR_1 in the crosstalk between coagulation, inflammation and fibrosis. In addition, clinical evidence also supports a role for PAR_1 in human fibrotic lung disease as this receptor has been found to be upregulated on

fibroblasts and macrophages within fibrotic foci in the lungs of patients with IPF (Howell *et al.*, 2005), ALI/ARDS (Howell *et al*, unpublished) and pulmonary fibrosis associated with systemic sclerosis (Bogatkevich *et al.*, 2005). Moreover, PAR_1 belongs to family of GPCRs that mediate a great range of biological responses elicited by a remarkably diverse array of stimuli including growth factors, chemoattractants, proteinases, etc. GPCRs are the target for nearly 30% of all drugs currently available (Hopkins and Groom, 2002). Therefore, PAR_1 has become a very attractive drug target in fibrotic lung disease. Indeed, there are currently a number of peptidomimetic and non-peptide PAR_1 antagonists available for both experimental studies and for pharmaceutical use in humans (Please see Introduction 1.7.5).

The current studies and studies by others have shown that the effectiveness of PAR_1 tethered ligand antagonist is limited by the presence of other PARs (O'Brien *et al.*, 2000; Shi *et al.*, 2004; Lidington *et al.*, 2005; Kaneider *et al.*, 2007). This raises concerns in terms of the therapeutic potential of PAR_1 antagonists. It is well-known that GPCRs can form hetero-oligomers, and the constitutive or ligand-induced oligomerization of GPCRs can lead to signal amplification and transactivation (Milligan, 2001; George *et al.*, 2002). Therefore other strategies will be required in order to avoid transactivation between PARs and the activation of PAR heterodimers. In this regard, this thesis demonstrated the effectiveness of blocking thrombin-induced CCL2 release using the recently developed novel PAR_1 antagonist Q94 (Caden Biosciences). Q94 is an allosteric antagonist that requires PAR_1 to be activated which unlike the conventional PAR_1 antagonists. It blocks PAR_1 at the intracellular interaction site with $G\alpha_q$, therefore should not lead to the transactivation of other PARs and the tethered ligand will be free to interact with its receptor rather than being prevented by antagonist binding site occupancy. In addition, Q94 only blocks PAR_1 coupling to $G\alpha_q$ and therefore allows more selective targeting of some, but not all, PAR_1-mediated cellular responses. This may therefore change the concept of therapeutic intervention from targeting all the cellular responses to selectively targeting deleterious responses, while preserving other cellular events that are essential for normal function.

5.4.2 Therapeutic implications of CCL2 in pulmonary fibrosis

This thesis has shown a dramatic increase in CCL2 by fibroblasts following PAR_1 activation. CCL2 is an important chemokine and exerts its potent pro-inflammatory and profibrotic effects. CCL2 is not only produced by numerous cell types, including fibroblasts, but can in turn activate and mediate cellular effects of these cells via autocrine and paracrine mechanisms (see Table 1.3 in introduction). For example,

CCL2 stimulates fibroblast collagen production via the induction of TGF-β and a reduction in PGE_2 production (See introduction section 1.8.3). More importantly, CCR2 (major CCL2 receptor) deficiency has been shown to be protective not only in bleomycin-induced pulmonary fibrosis (Moore *et al.*, 2001), but also in fluorescein isothiocyanate (FITC)-induced lung fibrosis (Gharaee-Kermani *et al.*, 2003). These observations suggest that strategies aimed at interfering with CCL2 production may offer promise for therapeutic intervention in pulmonary fibrosis.

5.5 Future studies

5.5.1 Molecular mechanism of RhoA activation induced by PAR_1-$G\alpha_q$ in mouse lung fibroblasts

By using the recently developed novel PAR_1 antagonist Q94, which blocks PAR_1 at the intracellular interaction site with $G\alpha_q$ (Caden Biosciences), this thesis has demonstrated that PAR_1-induced CCL2 release is mediated via PAR_1 coupling to $G\alpha_q$ and the activation of RhoA/Rho kinase. This indicates that $G\alpha_q$ can activate and signal via RhoA/Rho kinase directly. However, the molecular mechanisms involved in RhoA activation induced by $G\alpha_q$ have not been addressed in great detail in this thesis.

Fundamentally, GEFs play a critical role in RhoA activation as the activation of RhoA requires its dissociation from the RhoA.GDP.GDI complex followed by GTP exchange mediated by GEFs (Mehta and Malik, 2006). It is well-established that RhoGEFs including mainly LARG, p115RhoGEF and PDZ-RhoGEF are involved in mediating $G\alpha_{12/13}$-induced RhoA activation. However, the role of GEFs in $G\alpha_q$-induced RhoA activation needs further investigation. A recent study has proposed that p63RhoGEF is a downstream effector of the $G\alpha_q$ and this study also demonstrated the crystal structure of the $G\alpha_q$-p63RhoGEF-RhoA complex (Lutz *et al.*, 2007). Nevertheless, this thesis proposes that p115RhoGEF might be involved in $G\alpha_q$-mediated RhoA activation. This notion is based on the observations that p115RhoGEF contributes to PLC-β-Ca^{2+}-dependent PKC-induced RhoA activation after thrombin stimulation (Holinstat *et al.*, 2003; Singh *et al.*, 2007). Further experiments to address this notion are now warranted.

5.5.2 Further delineation of the signalling pathways involved in thrombin-induced CCL2 release in primary human adult lung fibroblasts

The work presented in this thesis showed that thrombin at 10 nM induced pHALF CCL2 release via both PAR_1-dependent and PAR-independent mechanisms, whereas PAR_1 is the major receptor involved in this response of thrombin in MLFs. To further compare

the signalling pathways downstream of PAR_1 that are involved in thrombin (10 nM)-induced CCL2 mRNA and protein levels in both human and murine cells, further studies are required. First, to investigate the involvement of other signalling molecules in thrombin-induced pHALF CCL2 release, including PLC, Ca^{2+}, PKC and c-Raf that have been shown to mediate thrombin-induced CCL2 release in MLFs. Second, to investigate the effect of thrombin on pHALF CCL2 mRNA levels and downstream signalling pathways involved.

In addition, this thesis also showed that PAR_1 may transactivate PAR_2 when PAR_1 signalling is blocked by tethered ligand binding site occupancy with RWJ-58259. The mechanism by which PAR_1 transactivates PAR_2 can be further investigated. To this end, PAR_2 antagonist or PAR_2 siRNA can be employed to block PAR_2 signalling.

5.5.3 Mechanism of protection of PAR_1-$G\alpha_q$ signalling inhibition in pulmonary fibrosis

The data presented in this thesis have provided compelling evidence that PAR_1-$G\alpha_q$ interaction acts as a fundamental component involved in thrombin-induced CCL2 release *in vitro*. Further studies are needed to assess the contribution of the PAR_1-$G\alpha_q$ signalling pathway in pulmonary fibrosis *in vivo*. It has been reported that some patients given bleomycin as an anti-neoplastic drug, develop pulmonary fibrosis in an age- and concentration-dependent manner (Luna *et al.*, 1972; Adamson, 1976, 1984; Papiris *et al.*, 2005). Lung sections obtained from these patients revealed that bleomycin-induced lung injury pathologically resembles UIP, the histopathological pattern of IPF. The bleomycin-induced lung fibrosis model could therefore be employed to investigate the contribution of PAR_1-$G\alpha_q$ signalling pathway in pulmonary fibrosis.

The contribution of PAR_1-$G\alpha_q$ signalling pathway following bleomycin-induced lung injury could be elucidated by performing the following experiments. First, the effect of PAR_1-$G\alpha_q$ inhibition by Q94 could be assessed. To this end, subsequent inflammation and fibrosis would be measured. Second, PKC, MAPKs, RhoA, and Rho kinase activation in the lungs of wild-type, bleomycin-injured and Q94 instilled mice could be measured by quantitative immunohitochemistry. Third, in order to address whether selectively interfering with some of the PAR_1 signalling may hold promise for therapeutic intervention, the consequences of interruption on PAR_1-$G\alpha_q$ coupling could be compared with the outcome in PAR_1 knockout mice after bleomycin injury or in mice treated with RWJ-58259.

5.5.4 Molecular mechanisms of high expression of CCL2 in pulmonary fibrosis

Previous work conducted in our laboratory has shown that CCL2 expression is high in bleomycin-induced lung injury model. In addition, cultured primary human lung fibroblasts derived from fibrotic lesions from patients with IPF show greater ability to express CCL2 when stimulated (Standiford *et al.*, 1993) and the reason for this is unknown. A detailed study of CCL2 production by primary human lung fibroblasts could determine the reason why it is switched on in IPF. Experiments involved in this study would include the investigation of transcriptional and post-transcriptional mechanisms involved in CCL2 release, as well as the determination of the site of the abnormal regulation of CCL2 release by fibroblasts in IPF. Identifying these key processes will likely identify new approaches to develop better treatments for IPF.

REFERENCES

Abe, R., Donnelly, S.C., Peng, T., Bucala, R., and Metz, C.N. (2001). Peripheral blood fibrocytes: differentiation pathway and migration to wound sites. J Immunol *166*, 7556-7562.

Abubakar, K., Schmidt, B., Monkman, S., Webber, C., de, S.D., and Roberts, R. (1998). Heparin improves gas exchange during experimental acute lung injury in newborn piglets. Am J Respir Crit Care Med *158*, 1620-1625.

Adamson, I.Y. (1976). Pulmonary toxicity of bleomycin. Environ Health Perspect *16*, 119-125.

Adamson, I.Y. (1984). Drug-induced pulmonary fibrosis. Environ Health Perspect *55*, 25-36.

Adamson, I.Y., Young, L., and Bowden, D.H. (1988). Relationship of alveolar epithelial injury and repair to the induction of pulmonary fibrosis. Am J Pathol *130*, 377-383.

Amano, M., Ito, M., Kimura, K., Fukata, Y., Chihara, K., Nakano, T., Matsuura, Y., and Kaibuchi, K. (1996). Phosphorylation and activation of myosin by Rho-associated kinase (Rho-kinase). J Biol Chem *271*, 20246-20249.

American Thoracic Society. (2000). Idiopathic pulmonary fibrosis: diagnosis and treatment. International consensus statement. American Thoracic Society (ATS), and the European Respiratory Society (ERS). Am J Respir Crit Care Med *161*, 646-664.

Amin, M.A., Mansfield, P.J., Pakozdi, A., Campbell, P.L., Ahmed, S., Martinez, R.J., and Koch, A.E. (2007). Interleukin-18 induces angiogenic factors in rheumatoid arthritis synovial tissue fibroblasts via distinct signaling pathways. Arthritis Rheum *56*, 1787-1797.

Andrade-Gordon, P., Derian, C.K., Maryanoff, B.E., Zhang, H.C., Addo, M.F., Cheung, W., Damiano, B.P., D'Andrea, M.R., Darrow, A.L., de Garavilla, L., Eckardt, A.J., Giardino, E.C., Haertlein, B.J., and McComsey, D.F. (2001). Administration of a potent antagonist of protease-activated receptor-1 (PAR-1) attenuates vascular restenosis following balloon angioplasty in rats. J Pharmacol Exp Ther *298*, 34-42.

Andrade-Gordon, P., Maryanoff, B.E., Derian, C.K., Zhang, H.C., Addo, M.F., Darrow, A.L., Eckardt, A.J., Hoekstra, W.J., McComsey, D.F., Oksenberg, D., Reynolds, E.E., Santulli, R.J., Scarborough, R.M., Smith, C.E., and White, K.B. (1999). Design, synthesis, and biological characterization of a peptide-mimetic antagonist for a tethered-ligand receptor. Proc Natl Acad Sci U S A *96*, 12257-12262.

Antoniades, H.N., Neville-Golden, J., Galanopoulos, T., Kradin, R.L., Valente, A.J., and Graves, D.T. (1992). Expression of monocyte chemoattractant protein 1 mRNA in human idiopathic pulmonary fibrosis. Proc Natl Acad Sci U S A *89*, 5371-5375.

Antoniou, K.M., Tzortzaki, E.G., Alexandrakis, M.G., Zervou, M., Tzanakis, N., Sfiridaki, K., Bouros, D.E., and Siafakas, N.M. (2005). Investigation of IL-18 and IL-12 in induced sputum of patients with IPF before and after treatment with interferon gamma-1b. Sarcoidosis Vasc Diffuse Lung Dis *22*, 204-209.

Anwar, K.N., Fazal, F., Malik, A.B., and Rahman, A. (2004). RhoA/Rho-associated kinase pathway selectively regulates thrombin-induced intercellular adhesion molecule-1 expression in endothelial cells via activation of I kappa B kinase beta and phosphorylation of RelA/p65. J Immunol *173*, 6965-6972.

Asokananthan, N., Graham, P.T., Fink, J., Knight, D.A., Bakker, A.J., McWilliam, A.S., Thompson, P.J., and Stewart, G.A. (2002). Activation of protease-activated receptor (PAR)-1, PAR-2, and PAR-4 stimulates IL-6, IL-8, and prostaglandin E2 release from human respiratory epithelial cells. J Immunol *168*, 3577-3585.

Axmann, A., Seidel, D., Reimann, T., Hempel, U., and Wenzel, K.W. (1998). Transforming growth factor-beta1-induced activation of the Raf-MEK-MAPK signaling pathway in rat lung fibroblasts via a PKC-dependent mechanism. Biochem Biophys Res Commun *249*, 456-460.

Azuma, A., Nukiwa, T., Tsuboi, E., Suga, M., Abe, S., Nakata, K., Taguchi, Y., Nagai, S., Itoh, H., Ohi, M., Sato, A., and Kudoh, S. (2005). Double-blind, placebo-controlled trial of pirfenidone in patients with idiopathic pulmonary fibrosis. Am J Respir Crit Care Med *171*, 1040-1047.

Babich, M., King, K.L., and Nissenson, R.A. (1990). Thrombin stimulates inositol phosphate production and intracellular free calcium by a pertussis toxin-insensitive mechanism in osteosarcoma cells. Endocrinology *126*, 948-954.

Bachhuber, B.G., Sarembock, I.J., Gimple, L.W., and Owens, G.K. (1997). alpha-Thrombin induces transforming growth factor-beta1 mRNA and protein in cultured vascular smooth muscle cells via a proteolytically activated receptor. J Vasc Res *34*, 41-48.

Bachli, E.B., Pech, C.M., Johnson, K.M., Johnson, D.J., Tuddenham, E.G., and McVey, J.H. (2003). Factor Xa and thrombin, but not factor VIIa, elicit specific cellular responses in dermal fibroblasts. J Thromb Haemost *1*, 1935-1944.

Baffy, G., Yang, L., Raj, S., Manning, D.R., and Williamson, J.R. (1994). G protein coupling to the thrombin receptor in Chinese hamster lung fibroblasts. J Biol Chem *269*, 8483-8487.

Bain, J., McLauchlan, H., Elliott, M., and Cohen, P. (2003). The specificities of protein kinase inhibitors: an update. Biochem J *371*, 199-204.

Bain, J., Plater, L., Elliott, M., Shpiro, N., Hastie, C.J., Mclauchlan, H., Klevernic, I., Arthur, J.S.C., Alessi, D.R., and Cohen, P. (2007). The selectivity of protein kinase inhibitors: a further update. Biochem J *408*, 297-315.

Barbas-Filho, J.V., Ferreira, M.A., Sesso, A., Kairalla, R.A., Carvalho, C.R., and Capelozzi, V.L. (2001). Evidence of type II pneumocyte apoptosis in the pathogenesis of idiopathic pulmonary fibrosis (IFP)/usual interstitial pneumonia (UIP). J Clin Pathol *54*, 132-138.

Bartha, K., Brisson, C., Archipoff, G., de la Salle, C., Lanza, F., Cazenave, J.P., and Beretz, A. (1993). Thrombin regulates tissue factor and thrombomodulin mRNA levels and activities in human saphenous vein endothelial cells by distinct mechanisms. J Biol Chem *268*, 421-429.

Baumgartner, K.B., Samet, J.M., Stidley, C.A., Colby, T.V., and Waldron, J.A. (1997). Cigarette smoking: a risk factor for idiopathic pulmonary fibrosis. Am J Respir Crit Care Med *155*, 242-248.

BelAiba, R.S., Djordjevic, T., Bonello, S., Artunc, F., Lang, F., Hess, J., and Gorlach, A. (2006). The serum- and glucocorticoid-inducible kinase Sgk-1 is involved in pulmonary vascular remodeling: role in redox-sensitive regulation of tissue factor by thrombin. Circ Res *98*, 828-836.

Bennett, B.L. (2006). c-Jun N-terminal kinase-dependent mechanisms in respiratory disease. Eur Respir J *28*, 651-661.

Berridge, M.J. (1993). Inositol trisphosphate and calcium signalling. Nature *361*, 315-325.

Bijli, K.M., Fazal, F., Minhajuddin, M., and Rahman, A. (2008). Activation of Syk by Protein Kinase C-{delta} Regulates Thrombin-induced Intercellular Adhesion Molecule-1 Expression in Endothelial Cells via Tyrosine Phosphorylation of RelA/p65. J Biol Chem *283*, 14674-14684.

Bilodeau, M.L., and Hamm, H.E. (2007). Regulation of protease-activated receptor (PAR) 1 and PAR4 signaling in human platelets by compartmentalized cyclic nucleotide actions. J Pharmacol Exp Ther *322*, 778-788.

Birnboim, H.C. (1983). A rapid alkaline extraction method for the isolation of plasmid DNA. Methods Enzymol *100*, 243-255.

Birukova, A.A., Birukov, K.G., Smurova, K., Adyshev, D., Kaibuchi, K., Alieva, I., Garcia, J.G., and Verin, A.D. (2004a). Novel role of microtubules in thrombin-induced endothelial barrier dysfunction. FASEB J *18*, 1879-1890.

Birukova, A.A., Smurova, K., Birukov, K.G., Kaibuchi, K., Garcia, J.G., and Verin, A.D. (2004b). Role of Rho GTPases in thrombin-induced lung vascular endothelial cells barrier dysfunction. Microvasc Res *67*, 64-77.

Blanc-Brude, O.P., Archer, F., Leoni, P., Derian, C., Bolsover, S., Laurent, G.J., and Chambers, R.C. (2005). Factor Xa stimulates fibroblast procollagen production, proliferation, and calcium signaling via PAR1 activation. Exp Cell Res *304*, 16-27.

Blanc-Brude, O.P., Chambers, R.C., Leoni, P., Dik, W.A., and Laurent, G.J. (2001). Factor Xa is a fibroblast mitogen via binding to effector-cell protease receptor-1 and autocrine release of PDGF. Am J Physiol Cell Physiol *281*, C681-689.

Blumer, K.J., and Johnson, G.L. (1994). Diversity in function and regulation of MAP kinase pathways. Trends Biochem Sci *19*, 236-240.

Bogatkevich, G.S., Gustilo, E., Oates, J.C., Feghali-Bostwick, C., Harley, R.A., Silver, R.M., and Ludwicka-Bradley, A. (2005). Distinct PKC isoforms mediate cell survival and DNA synthesis in thrombin-induced myofibroblasts. Am J Physiol Lung Cell Mol Physiol *288*, L190-201.

Bogatkevich, G.S., Tourkina, E., Abrams, C.S., Harley, R.A., Silver, R.M., and Ludwicka-Bradley, A. (2003). Contractile activity and smooth muscle alpha-actin organization in thrombin-induced human lung myofibroblasts. Am J Physiol Lung Cell Mol Physiol *285*, L334-343.

Bogatkevich, G.S., Tourkina, E., Silver, R.M., and Ludwicka-Bradley, A. (2001). Thrombin differentiates normal lung fibroblasts to a myofibroblast phenotype via the proteolytically activated receptor-1 and a protein kinase C-dependent pathway. J Biol Chem *276*, 45184-45192.

Bohm, S.K., Khitin, L.M., Grady, E.F., Aponte, G., Payan, D.G., and Bunnett, N.W. (1996). Mechanisms of desensitization and resensitization of proteinase-activated receptor-2. J Biol Chem *271*, 22003-22016.
Boire, A., Covic, L., Agarwal, A., Jacques, S., Sherifi, S., and Kuliopulos, A. (2005). PAR1 is a matrix metalloprotease-1 receptor that promotes invasion and tumorigenesis of breast cancer cells. Cell *120*, 303-313.

Bombara, M.P., Webb, D.L., Conrad, P., Marlor, C.W., Sarr, T., Ranges, G.E., Aune, T.M., Greve, J.M., and Blue, M.L. (1993). Cell contact between T cells and synovial fibroblasts causes induction of adhesion molecules and cytokines. J Leukoc Biol *54*, 399-406.

Bonfield, T.L., Panuska, J.R., Konstan, M.W., Hilliard, K.A., Hilliard, J.B., Ghnaim, H., and Berger, M. (1995). Inflammatory cytokines in cystic fibrosis lungs. Am J Respir Crit Care Med *152*, 2111-2118.

Bonniaud, P., Margetts, P.J., Kolb, M., Haberberger, T., Kelly, M., Robertson, J., and Gauldie, J. (2003). Adenoviral gene transfer of connective tissue growth factor in the lung induces transient fibrosis. Am J Respir Crit Care Med *168*, 770-778.

Booden, M.A., Siderovski, D.P., and Der, C.J. (2002). Leukemia-associated Rho guanine nucleotide exchange factor promotes G alpha q-coupled activation of RhoA. Mol Cell Biol *22*, 4053-4061.

Borbiev, T., Birukova, A., Liu, F., Nurmukhambetova, S., Gerthoffer, W.T., Garcia, J.G., and Verin, A.D. (2004). p38 MAP kinase-dependent regulation of endothelial cell permeability. Am J Physiol Lung Cell Mol Physiol *287*, L911-918.

Borchers, M.T., Biechele, T., Justice, J.P., Ansay, T., Cormier, S., Mancino, V., Wilkie, T.M., Simon, M.I., Lee, N.A., and Lee, J.J. (2003). Methacholine-induced airway hyperresponsiveness is dependent on Galphaq signaling. Am J Physiol Lung Cell Mol Physiol *285*, L114-120.

Borsello, T., Clarke, P.G., Hirt, L., Vercelli, A., Repici, M., Schorderet, D.F., Bogousslavsky, J., and Bonny, C. (2003). A peptide inhibitor of c-Jun N-terminal kinase protects against excitotoxicity and cerebral ischemia. Nat Med *9*, 1180-1186.

Boulton, T.G., Nye, S.H., Robbins, D.J., Ip, N.Y., Radziejewska, E., Morgenbesser, S.D., DePinho, R.A., Panayotatos, N., Cobb, M.H., and Yancopoulos, G.D. (1991). ERKs: a family of protein-serine/threonine kinases that are activated and tyrosine phosphorylated in response to insulin and NGF. Cell *65*, 663-675.

Brandes, R.P., Viedt, C., Nguyen, K., Beer, S., Kreuzer, J., Busse, R., and Gorlach, A. (2001). Thrombin-induced MCP-1 expression involves activation of the p22phox-containing NADPH oxidase in human vascular smooth muscle cells. Thromb Haemost *85*, 1104-1110.

Brass, L.F., Pizarro, S., Ahuja, M., Belmonte, E., Blanchard, N., Stadel, J.M., and Hoxie, J.A. (1994). Changes in the structure and function of the human thrombin receptor during receptor activation, internalization, and recycling. J Biol Chem *269*, 2943-2952.

Bringardner, B.D., Baran, C.P., Eubank, T.D., and Marsh, C.B. (2008). The role of inflammation in the pathogenesis of idiopathic pulmonary fibrosis. Antioxid Redox Signal *10*, 287-302.

Broekelmann, T.J., Limper, A.H., Colby, T.V., and McDonald, J.A. (1991). Transforming growth factor beta 1 is present at sites of extracellular matrix gene expression in human pulmonary fibrosis. Proc Natl Acad Sci U S A *88*, 6642-6646.
Bucala, R., Spiegel, L.A., Chesney, J., Hogan, M., and Cerami, A. (1994). Circulating fibrocytes define a new leukocyte subpopulation that mediates tissue repair. Mol Med *1*, 71-81.

Burysek, L., Syrovets, T., and Simmet, T. (2002). The serine protease plasmin triggers expression of MCP-1 and CD40 in human primary monocytes via activation of p38 MAPK and janus kinase (JAK)/STAT signaling pathways. J Biol Chem *277*, 33509-33517.

Bussfeld, D., Nain, M., Hofmann, P., Gemsa, D., and Sprenger, H. (2000). Selective induction of the monocyte-attracting chemokines MCP-1 and IP-10 in vesicular stomatitis virus-infected human monocytes. J Interferon Cytokine Res *20*, 615-621.

Calderon, T.M., Eugenin, E.A., Lopez, L., Kumar, S.S., Hesselgesser, J., Raine, C.S., and Berman, J.W. (2006). A role for CXCL12 (SDF-1alpha) in the pathogenesis of multiple sclerosis: regulation of CXCL12 expression in astrocytes by soluble myelin basic protein. J Neuroimmunol *177*, 27-39.

Camerer, E. (2007). Unchecked thrombin is bad news for troubled arteries. J Clin Invest *117*, 1486-1489.

Carlile-Klusacek, M., and Rizzo, V. (2007). Endothelial cytoskeletal reorganization in response to PAR1 stimulation is mediated by membrane rafts but not caveolae. Am J Physiol Heart Circ Physiol *293*, H366-375.

Chackalamannil, S., Xia, Y., Greenlee, W.J., Clasby, M., Doller, D., Tsai, H., Asberom, T., Czarniecki, M., Ahn, H.S., Boykow, G., Foster, C., Agans-Fantuzzi, J., Bryant, M., Lau, J., and Chintala, M. (2005). Discovery of potent orally active thrombin receptor (protease activated receptor 1) antagonists as novel antithrombotic agents. J Med Chem *48*, 5884-5887.

Chalmers, C.J., Balmanno, K., Hadfield, K., Ley, R., and Cook, S.J. (2003). Thrombin inhibits Bim (Bcl-2-interacting mediator of cell death) expression and prevents serum-withdrawal-induced apoptosis via protease-activated receptor 1. Biochem J *375*, 99-109.

Chambers, R.C. (2003). Role of coagulation cascade proteases in lung repair and fibrosis. Eur Respir J Suppl *44*, 33s-35s.

Chambers, R.C. (2008). Procoagulant signalling mechanisms in lung inflammation and fibrosis: novel opportunities for pharmacological intervention? Br J Pharmacol *153 Suppl 1*, S367-378.

Chambers, R.C., Dabbagh, K., McAnulty, R.J., Gray, A.J., Blanc-Brude, O.P., and Laurent, G.J. (1998). Thrombin stimulates fibroblast procollagen production via proteolytic activation of protease-activated receptor 1. Biochem J *333 (Pt 1)*, 121-127.

Chambers, R.C., and Laurent, G.J. (2002). Coagulation cascade proteases and tissue fibrosis. Biochem Soc Trans *30*, 194-200.

Chambers, R.C., Leoni, P., Blanc-Brude, O.P., Wembridge, D.E., and Laurent, G.J. (2000). Thrombin is a potent inducer of connective tissue growth factor production via proteolytic activation of protease-activated receptor-1. J Biol Chem *275*, 35584-35591.
Chandler, D.B. (1990). Possible mechanisms of bleomycin-induced fibrosis. Clin Chest Med *11*, 21-30.

Chandrasekharan, U.M., Yang, L., Walters, A., Howe, P., and DiCorleto, P.E. (2004). Role of CL-100, a dual specificity phosphatase, in thrombin-induced endothelial cell activation. J Biol Chem *279*, 46678-46685.

Chapman, H.A., Allen, C.L., and Stone, O.L. (1986). Abnormalities in pathways of alveolar fibrin turnover among patients with interstitial lung disease. Am Rev Respir Dis *133*, 437-443.

Chelliah, M.V., Chackalamannil, S., Xia, Y., Eagen, K., Clasby, M.C., Gao, X., Greenlee, W., Ahn, H.S., Agans-Fantuzzi, J., Boykow, G., Hsieh, Y., Bryant, M., Palamanda, J., Chan, T.M., Hesk, D., and Chintala, M. (2007). Heterotricyclic himbacine analogs as potent, orally active thrombin receptor (protease activated receptor-1) antagonists. J Med Chem *50*, 5147-5160.

Cheung, W.M., D'Andrea, M.R., Andrade-Gordon, P., and Damiano, B.P. (1999). Altered vascular injury responses in mice deficient in protease-activated receptor-1. Arterioscler Thromb Vasc Biol *19*, 3014-3024.

Chiba, Y., and Misawa, M. (2001). Increased expression of G12 and G13 proteins in bronchial smooth muscle of airway hyperresponsive rats. Inflamm Res *50*, 333-336.

Chikumi, H., Vazquez-Prado, J., Servitja, J.M., Miyazaki, H., and Gutkind, J.S. (2002). Potent activation of RhoA by Galpha q and Gq-coupled receptors. J Biol Chem *277*, 27130-27134.

Chin, A.C., Vergnolle, N., MacNaughton, W.K., Wallace, J.L., Hollenberg, M.D., and Buret, A.G. (2003). Proteinase-activated receptor 1 activation induces epithelial apoptosis and increases intestinal permeability. Proc Natl Acad Sci U S A *100*, 11104-11109.

Christensen, P.J., Goodman, R.E., Pastoriza, L., Moore, B., and Toews, G.B. (1999). Induction of lung fibrosis in the mouse by intratracheal instillation of fluorescein isothiocyanate is not T-cell-dependent. Am J Pathol *155*, 1773-1779.

Chung, K.F. (2001). Cytokines in chronic obstructive pulmonary disease. Eur Respir J Suppl *34*, 50s-59s.

Cobb, M.H., Xu, S., Hepler, J.E., Hutchison, M., Frost, J., and Robbins, D.J. (1994). Regulation of the MAP kinase cascade. Cell Mol Biol Res *40*, 253-256.

Cochran, B.H., Reffel, A.C., and Stiles, C.D. (1983). Molecular cloning of gene sequences regulated by platelet-derived growth factor. Cell *33*, 939-947.

Cohen, P. (1999). The development and therapeutic potential of protein kinase inhibitors. Curr Opin Chem Biol *3*, 459-465.

Collard, H.R., and King, T.E., Jr. (2001). Treatment of idiopathic pulmonary fibrosis: the rise and fall of corticosteroids. Am J Med *110*, 326-328.

Colotta, F., Borre, A., Wang, J.M., Tattanelli, M., Maddalena, F., Polentarutti, N., Peri, G., and Mantovani, A. (1992). Expression of a monocyte chemotactic cytokine by human mononuclear phagocytes. J Immunol *148*, 760-765.

Colotta, F., Sciacca, F.L., Sironi, M., Luini, W., Rabiet, M.J., and Mantovani, A. (1994). Expression of monocyte chemotactic protein-1 by monocytes and endothelial cells exposed to thrombin. Am J Pathol *144*, 975-985.

Coughlin, S.R. (1999). Protease-activated receptors and platelet function. Thromb Haemost *82*, 353-356.

Coughlin, S.R. (2000). Thrombin signalling and protease-activated receptors. Nature *407*, 258-264.

Coultas, D.B., Zumwalt, R.E., Black, W.C., and Sobonya, R.E. (1994). The epidemiology of interstitial lung diseases. Am J Respir Crit Care Med *150*, 967-972.

Covic, L., Gresser, A.L., Talavera, J., Swift, S., and Kuliopulos, A. (2002a). Activation and inhibition of G protein-coupled receptors by cell-penetrating membrane-tethered peptides. Proc Natl Acad Sci U S A *99*, 643-648.

Covic, L., Misra, M., Badar, J., Singh, C., and Kuliopulos, A. (2002b). Pepducin-based intervention of thrombin-receptor signaling and systemic platelet activation. Nat Med *8*, 1161-1165.

Cowley, S., Paterson, H., Kemp, P., and Marshall, C.J. (1994). Activation of MAP kinase kinase is necessary and sufficient for PC12 differentiation and for transformation of NIH 3T3 cells. Cell *77*, 841-852.

Cuenda, A., Rouse, J., Doza, Y.N., Meier, R., Cohen, P., Gallagher, T.F., Young, P.R., and Lee, J.C. (1995). SB 203580 is a specific inhibitor of a MAP kinase homologue which is stimulated by cellular stresses and interleukin-1. FEBS Lett *364*, 229-233.

Cunningham, M.A., Rondeau, E., Chen, X., Coughlin, S.R., Holdsworth, S.R., and Tipping, P.G. (2000). Protease-activated receptor 1 mediates thrombin-dependent, cell-mediated renal inflammation in crescentic glomerulonephritis. J Exp Med *191*, 455-462.

Cunningham, P.N., Hack, B.K., Ren, G., Minto, A.W., Morgan, B.P., and Quigg, R.J. (2001). Glomerular complement regulation is overwhelmed in passive Heymann nephritis. Kidney Int *60*, 900-909.

Damiano, B.P., Derian, C.K., Maryanoff, B.E., Zhang, H.C., and Gordon, P.A. (2003). RWJ-58259: a selective antagonist of protease activated receptor-1. Cardiovasc Drug Rev *21*, 313-326.

Davie, E.W., and Kulman, J.D. (2006). An overview of the structure and function of thrombin. Semin Thromb Hemost *32 Suppl 1*, 3-15.

Davies, S.P., Reddy, H., Caivano, M., and Cohen, P. (2000). Specificity and mechanism of action of some commonly used protein kinase inhibitors. Biochem J *351*, 95-105.

Davis, R.J. (2000). Signal transduction by the JNK group of MAP kinases. Cell *103*, 239-252.

Dawes, K.E., Gray, A.J., and Laurent, G.J. (1993). Thrombin stimulates fibroblast chemotaxis and replication. Eur J Cell Biol *61*, 126-130.

de Boer, W.I., Alagappan, V.K., and Sharma, H.S. (2007). Molecular mechanisms in chronic obstructive pulmonary disease: potential targets for therapy. Cell Biochem Biophys *47*, 131-148.

Della Rocca, G.J., Maudsley, S., Daaka, Y., Lefkowitz, R.J., and Luttrell, L.M. (1999). Pleiotropic coupling of G protein-coupled receptors to the mitogen-activated protein kinase cascade. Role of focal adhesions and receptor tyrosine kinases. J Biol Chem *274*, 13978-13984.

Demedts, M., Behr, J., Buhl, R., Costabel, U., Dekhuijzen, R., Jansen, H.M., MacNee, W., Thomeer, M., Wallaert, B., Laurent, F., Nicholson, A.G., Verbeken, E.K., Verschakelen, J., Flower, C.D., Capron, F., Petruzzelli, S., De Vuyst, P., van den Bosch, J.M., Rodriguez-Becerra, E., Corvasce, G., Lankhorst, I., Sardina, M., and Montanari, M. (2005). High-dose acetylcysteine in idiopathic pulmonary fibrosis. N Engl J Med *353*, 2229-2242.

Dempsey, E.C., Cool, C.D., and Littler, C.M. (2007). Lung disease and PKCs. Pharmacol Res *55*, 545-559.

Dempsey, E.C., Newton, A.C., Mochly-Rosen, D., Fields, A.P., Reyland, M.E., Insel, P.A., and Messing, R.O. (2000). Protein kinase C isozymes and the regulation of diverse cell responses. Am J Physiol Lung Cell Mol Physiol *279*, L429-438.

Derian, C.K., Damiano, B.P., Addo, M.F., Darrow, A.L., D'Andrea, M.R., Nedelman, M., Zhang, H.C., Maryanoff, B.E., and Andrade-Gordon, P. (2003). Blockade of the thrombin receptor protease-activated receptor-1 with a small-molecule antagonist prevents thrombus formation and vascular occlusion in nonhuman primates. J Pharmacol Exp Ther *304*, 855-861.

Dery, O., Corvera, C.U., Steinhoff, M., and Bunnett, N.W. (1998). Proteinase-activated receptors: novel mechanisms of signaling by serine proteases. Am J Physiol *274*, C1429-1452.

Di Serio, C., Pellerito, S., Duarte, M., Massi, D., Naldini, A., Cirino, G., Prudovsky, I., Santucci, M., Geppetti, P., Marchionni, N., Masotti, G., and Tarantini, F. (2007). Protease-activated receptor 1-selective antagonist SCH79797 inhibits cell proliferation and induces apoptosis by a protease-activated receptor 1-independent mechanism. Basic Clin Pharmacol Toxicol *101*, 63-69.

Diaz, A., and Jimenez, S.A. (1997). Interferon-gamma regulates collagen and fibronectin gene expression by transcriptional and post-transcriptional mechanisms. Int J Biochem Cell Biol *29*, 251-260.

Dik, W.A., Zimmermann, L.J., Naber, B.A., Janssen, D.J., van Kaam, A.H., and Versnel, M.A. (2003). Thrombin contributes to bronchoalveolar lavage fluid mitogenicity in lung disease of the premature infant. Pediatr Pulmonol *35*, 34-41.

Du, J., Brink, M., Peng, T., Mottironi, B., and Delafontaine, P. (2001). Thrombin regulates insulin-like growth factor-1 receptor transcription in vascular smooth muscle: characterization of the signaling pathway. Circ Res *88*, 1044-1052.

Eickelberg, O., Kohler, E., Reichenberger, F., Bertschin, S., Woodtli, T., Erne, P., Perruchoud, A.P., and Roth, M. (1999). Extracellular matrix deposition by primary human lung fibroblasts in response to TGF-beta1 and TGF-beta3. Am J Physiol *276*, L814-824.

Eidt, J.F., Allison, P., Noble, S., Ashton, J., Golino, P., McNatt, J., Buja, L.M., and Willerson, J.T. (1988). Thrombin is an important mediator of cyclic coronary blood flow variations due to platelet aggregation in stenosed canine coronary arteries. Trans Assoc Am Physicians *101*, 125-136.

Ellis, C.A., Malik, A.B., Gilchrist, A., Hamm, H., Sandoval, R., Voyno-Yasenetskaya, T., and Tiruppathi, C. (1999). Thrombin induces proteinase-activated receptor-1 gene expression in endothelial cells via activation of Gi-linked Ras/mitogen-activated protein kinase pathway. J Biol Chem *274*, 13718-13727.

Emad, A., and Emad, V. (2007). Elevated levels of MCP-1, MIP-alpha and MIP-1 beta in the bronchoalveolar lavage (BAL) fluid of patients with mustard gas-induced pulmonary fibrosis. Toxicology *240*, 60-69.

Etienne-Manneville, S., and Hall, A. (2002). Rho GTPases in cell biology. Nature *420*, 629-635.

Faller, D.V., Weng, H., Graves, D.T., and Choi, S.Y. (1997). Moloney murine leukemia virus long terminal repeat activates monocyte chemotactic protein-1 protein expression and chemotactic activity. J Cell Physiol *172*, 240-252.

Fang, Q., Liu, X., Abe, S., Kobayashi, T., Wang, X.Q., Kohyama, T., Hashimoto, M., Wyatt, T., and Rennard, S.I. (2004). Thrombin induces collagen gel contraction partially through PAR1 activation and PKC-epsilon. Eur Respir J *24*, 918-924.

Fang, Q., Mao, L., Kobayashi, T., Wang, X., Wyatt, T.A., Kim, H., Liu, X., and Rennard, S.I. (2008). PKCdelta mediates thrombin-augmented fibroblast-mediated collagen gel contraction. Biochem Biophys Res Commun *369*, 1199-1203.

Fattman, C.L. (2008). Apoptosis in pulmonary fibrosis: too much or not enough? Antioxid Redox Signal *10*, 379-386.

Feistritzer, C., and Riewald, M. (2005). Endothelial barrier protection by activated protein C through PAR1-dependent sphingosine 1-phosphate receptor-1 crossactivation. Blood *105*, 3178-3184.

Feliciello, A., Gottesman, M.E., and Avvedimento, E.V. (2001). The biological functions of A-kinase anchor proteins. J Mol Biol *308*, 99-114.

Fields, T.A., and Casey, P.J. (1997). Signalling functions and biochemical properties of pertussis toxin-resistant G-proteins. Biochem J *321 (Pt 3)*, 561-571.

Finzer, P., Soto, U., Delius, H., Patzelt, A., Coy, J.F., Poustka, A., zur Hausen, H., and Rosl, F. (2000). Differential transcriptional regulation of the monocyte-chemoattractant protein-1 (MCP-1) gene in tumorigenic and non-tumorigenic HPV 18 positive cells: the role of the chromatin structure and AP-1 composition. Oncogene *19*, 3235-3244.

Fiorucci, S., Antonelli, E., Distrutti, E., Severino, B., Fiorentina, R., Baldoni, M., Caliendo, G., Santagada, V., Morelli, A., and Cirino, G. (2004). PAR1 antagonism protects against experimental liver fibrosis. Role of proteinase receptors in stellate cell activation. Hepatology *39*, 365-375.

Flaherty, D., Hinde, S., Monick, M., Powers, L., Bradford, M., Yarovinsky, T., and Hunninghake, G. (2004). Adenovirus vectors activate survival pathways in lung epithelial cells. Am J Physiol Lung Cell Mol Physiol *287*, L393-401.

Flaherty, D.M., Monick, M.M., and Hinde, S.L. (2006). Human alveolar macrophages are deficient in PTEN. The role of endogenous oxidants. J Biol Chem *281*, 5058-5064.

Flaherty, K., Toews, G., Lynch, J.r., Kazerooni, E., Gross, B., Strawderman, R., Hariharan, K., Flint, A., and Martinez, F. (2001a). Steroids in idiopathic pulmonary fibrosis: a prospective assessment of adverse reactions, response to therapy, and survival. Am J Med *110*, 278-282.

Flaherty, K.R., Travis, W.D., Colby, T.V., Toews, G.B., Kazerooni, E.A., Gross, B.H., Jain, A., Strawderman, R.L., Flint, A., Lynch, J.P., and Martinez, F.J. (2001b). Histopathologic variability in usual and nonspecific interstitial pneumonias. Am J Respir Crit Care Med *164*, 1722-1727.

Frankel, S.K., Cosgrove, G.P., Cha, S.I., Cool, C.D., Wynes, M.W., Edelman, B.L., Brown, K.K., and Riches, D.W. (2006). TNF-alpha sensitizes normal and fibrotic human lung fibroblasts to Fas-induced apoptosis. Am J Respir Cell Mol Biol *34*, 293-304.

Frazier-Jessen, M.R., and Kovacs, E.J. (1995). Estrogen modulation of JE/monocyte chemoattractant protein-1 mRNA expression in murine macrophages. J Immunol *154*, 1838-1845.

Fujita, M., Shannon, J.M., Morikawa, O., Gauldie, J., Hara, N., and Mason, R.J. (2003). Overexpression of tumor necrosis factor-alpha diminishes pulmonary fibrosis induced by bleomycin or transforming growth factor-beta. Am J Respir Cell Mol Biol *29*, 669-676.

Fukuhara, S., Chikumi, H., and Gutkind, J.S. (2001). RGS-containing RhoGEFs: the missing link between transforming G proteins and Rho? Oncogene *20*, 1661-1668.

Fukumoto, Y., Tawara, S., and Shimokawa, H. (2007). Recent progress in the treatment of pulmonary arterial hypertension: expectation for rho-kinase inhibitors. Tohoku J Exp Med *211*, 309-320.

Gabazza, E.C., Taguchi, O., Tamaki, S., Takeya, H., Kobayashi, H., Yasui, H., Kobayashi, T., Hataji, O., Urano, H., Zhou, H., Suzuki, K., and Adachi, Y. (1999). Thrombin in the airways of asthmatic patients. Lung *177*, 253-262.

Gando, S., Kameue, T., Matsuda, N., Hayakawa, M., Morimoto, Y., Ishitani, T., and Kemmotsu, O. (2003). Imbalances between the levels of tissue factor and tissue factor pathway inhibitor in ARDS patients. Thromb Res *109*, 119-124.

George, S.R., O'Dowd, B.F., and Lee, S.P. (2002). G-protein-coupled receptor oligomerization and its potential for drug discovery. Nat Rev Drug Discov *1*, 808-820.

Gharaee-Kermani, M., Denholm, E.M., and Phan, S.H. (1996). Costimulation of fibroblast collagen and transforming growth factor beta1 gene expression by monocyte chemoattractant protein-1 via specific receptors. J Biol Chem *271*, 17779-17784.

Gharaee-Kermani, M., Gyetko, M.R., Hu, B., and Phan, S.H. (2007). New insights into the pathogenesis and treatment of idiopathic pulmonary fibrosis: a potential role for stem cells in the lung parenchyma and implications for therapy. Pharm Res *24*, 819-841.

Gharaee-Kermani, M., McCullumsmith, R.E., Charo, I.F., Kunkel, S.L., and Phan, S.H. (2003). CC-chemokine receptor 2 required for bleomycin-induced pulmonary fibrosis. Cytokine *24*, 266-276.

Gharaee-Kermani, M., and Phan, S.H. (2001). Role of cytokines and cytokine therapy in wound healing and fibrotic diseases. Curr Pharm Des *7*, 1083-1103.

Gilchrist, A., Li, A., and Hamm, H.E. (2002). Design and use of C-terminal minigene vectors for studying role of heterotrimeric G proteins. Methods Enzymol *344*, 58-69.

Gilchrist, A., Mazzoni, M.R., Dineen, B., Dice, A., Linden, J., Proctor, W.R., Lupica, C.R., Dunwiddie, T.V., and Hamm, H.E. (1998). Antagonists of the receptor-G protein interface block Gi-coupled signal transduction. J Biol Chem *273*, 14912-14919.

Gilchrist, A., Vanhauwe, J.F., Li, A., Thomas, T.O., Voyno-Yasenetskaya, T., and Hamm, H.E. (2001). G alpha minigenes expressing C-terminal peptides serve as specific inhibitors of thrombin-mediated endothelial activation. J Biol Chem *276*, 25672-25679.

Glabinski, A.R., Balasingam, V., Tani, M., Kunkel, S.L., Strieter, R.M., Yong, V.W., and Ransohoff, R.M. (1996). Chemokine monocyte chemoattractant protein-1 is expressed by astrocytes after mechanical injury to the brain. J Immunol *156*, 4363-4368.

Goebeler, M., Gillitzer, R., Kilian, K., Utzel, K., Brocker, E.B., Rapp, U.R., and Ludwig, S. (2001). Multiple signaling pathways regulate NF-kappaB-dependent transcription of the monocyte chemoattractant protein-1 gene in primary endothelial cells. Blood *97*, 46-55.

Goebeler, M., Kilian, K., Gillitzer, R., Kunz, M., Yoshimura, T., Brocker, E.B., Rapp, U.R., and Ludwig, S. (1999). The MKK6/p38 stress kinase cascade is critical for tumor necrosis factor-alpha-induced expression of monocyte-chemoattractant protein-1 in endothelial cells. Blood *93*, 857-865.

Goel, R., Phillips-Mason, P.J., Gardner, A., Raben, D.M., and Baldassare, J.J. (2004). Alpha-thrombin-mediated phosphatidylinositol 3-kinase activation through release of Gbetagamma dimers from Galphaq and Galphai2. J Biol Chem *279*, 6701-6710.

Gomperts, B.N., and Strieter, R.M. (2007). Fibrocytes in lung disease. J Leukoc Biol *82*, 449-456.

Goppelt-Struebe, M., and Stroebel, M. (1995). Synergistic induction of monocyte chemoattractant protein-1 (MCP-1) by platelet-derived growth factor and interleukin-1. FEBS Lett *374*, 375-378.

Grand, R.J., Turnell, A.S., and Grabham, P.W. (1996). Cellular consequences of thrombin-receptor activation. Biochem J *313 (Pt 2)*, 353-368.

Grandaliano, G., Choudhury, G.G., Biswas, P., and Abboud, H.E. (1994). Mitogenic signaling of thrombin in mesangial cells: role of tyrosine phosphorylation. Am J Physiol *267*, F528-536.

Grandaliano, G., Monno, R., Ranieri, E., Gesualdo, L., Schena, F.P., Martino, C., and Ursi, M. (2000). Regenerative and proinflammatory effects of thrombin on human proximal tubular cells. J Am Soc Nephrol *11*, 1016-1025.

Greenburg, G., and Hay, E.D. (1982). Epithelia suspended in collagen gels can lose polarity and express characteristics of migrating mesenchymal cells. J Cell Biol *95*, 333-339.

Gribbin, J., Hubbard, R.B., Le Jeune, I., Smith, C.J., West, J., and Tata, L.J. (2006). Incidence and mortality of idiopathic pulmonary fibrosis and sarcoidosis in the UK. Thorax *61*, 980-985.

Gu, L., Tseng, S., Horner, R.M., Tam, C., Loda, M., and Rollins, B.J. (2000). Control of TH2 polarization by the chemokine monocyte chemoattractant protein-1. Nature *404*, 407-411.

Guo, Z., Boekhoudt, G.H., and Boss, J.M. (2003). Role of the intronic enhancer in tumor necrosis factor-mediated induction of manganous superoxide dismutase. J Biol Chem *278*, 23570-23578.

Hall, R.A., Premont, R.T., and Lefkowitz, R.J. (1999). Heptahelical receptor signaling: beyond the G protein paradigm. J Cell Biol *145*, 927-932.

Hamm, H.E. (1998). The many faces of G protein signaling. J Biol Chem *273*, 669-672.
Hamm, H.E., and Gilchrist, A. (1996). Heterotrimeric G proteins. Curr Opin Cell Biol *8*, 189-196.

Hammes, S.R., and Coughlin, S.R. (1999). Protease-activated receptor-1 can mediate responses to SFLLRN in thrombin-desensitized cells: evidence for a novel mechanism for preventing or terminating signaling by PAR1's tethered ligand. Biochemistry *38*, 2486-2493.

Hart, C.E., Bailey, M., Curtis, D.A., Osborn, S., Raines, E., Ross, R., and Forstrom, J.W. (1990). Purification of PDGF-AB and PDGF-BB from human platelet extracts and identification of all three PDGF dimers in human platelets. Biochemistry *29*, 166-172.

Hart, M.J., Jiang, X., Kozasa, T., Roscoe, W., Singer, W.D., Gilman, A.G., Sternweis, P.C., and Bollag, G. (1998). Direct stimulation of the guanine nucleotide exchange activity of p115 RhoGEF by Galpha13. Science *280*, 2112-2114.

Haschek, W.M., and Witschi, H. (1979). Pulmonary fibrosis--a possible mechanism. Toxicol Appl Pharmacol *51*, 475-487.

Hayashi, A., Seki, N., Hattori, A., Kozuma, S., and Saito, T. (1999). PKCnu, a new member of the protein kinase C family, composes a fourth subfamily with PKCmu. Biochim Biophys Acta *1450*, 99-106.

Hein, L., Ishii, K., Coughlin, S.R., and Kobilka, B.K. (1994). Intracellular targeting and trafficking of thrombin receptors. A novel mechanism for resensitization of a G protein-coupled receptor. J Biol Chem *269*, 27719-27726.

Henn, V., Slupsky, J.R., Grafe, M., Anagnostopoulos, I., Forster, R., Muller-Berghaus, G., and Kroczek, R.A. (1998). CD40 ligand on activated platelets triggers an inflammatory reaction of endothelial cells. Nature *391*, 591-594.

Hernandez-Rodriguez, N.A., Cambrey, A.D., Harrison, N.K., Chambers, R.C., Gray, A.J., Southcott, A.M., duBois, R.M., Black, C.M., Scully, M.F., McAnulty, R.J., and et al. (1995). Role of thrombin in pulmonary fibrosis. Lancet *346*, 1071-1073.

Hetzel, M., Bachem, M., Anders, D., Trischler, G., and Faehling, M. (2005). Different effects of growth factors on proliferation and matrix production of normal and fibrotic human lung fibroblasts. Lung *183*, 225-237.

Hiol, A., Davey, P.C., Osterhout, J.L., Waheed, A.A., Fischer, E.R., Chen, C.K., Milligan, G., Druey, K.M., and Jones, T.L. (2003). Palmitoylation regulates regulators of G-protein signaling (RGS) 16 function. I. Mutation of amino-terminal cysteine residues on RGS16 prevents its targeting to lipid rafts and palmitoylation of an internal cysteine residue. J Biol Chem *278*, 19301-19308.

Hirano, F., Kobayashi, A., Hirano, Y., Nomura, Y., Fukawa, E., and Makino, I. (2002). Thrombin-induced expression of RANTES mRNA through protease activated receptor-1 in human synovial fibroblasts. Ann Rheum Dis *61*, 834-837.

Hodges, R.J., Jenkins, R.G., Wheeler-Jones, C.P., Copeman, D.M., Bottoms, S.E., Bellingan, G.J., Nanthakumar, C.B., Laurent, G.J., Hart, S.L., Foster, M.L., and McAnulty, R.J. (2004). Severity of lung injury in cyclooxygenase-2-deficient mice is dependent on reduced prostaglandin E(2) production. Am J Pathol *165*, 1663-1676.

Hogaboam, C.M., Lukacs, N.W., Chensue, S.W., Strieter, R.M., and Kunkel, S.L. (1998a). Monocyte chemoattractant protein-1 synthesis by murine lung fibroblasts modulates CD4+ T cell activation. J Immunol *160*, 4606-4614.

Hogaboam, C.M., Smith, R.E., and Kunkel, S.L. (1998b). Dynamic interactions between lung fibroblasts and leukocytes: implications for fibrotic lung disease. Proc Assoc Am Physicians *110*, 313-320.

Hogan, B.L., and Yingling, J.M. (1998). Epithelial/mesenchymal interactions and branching morphogenesis of the lung. Curr Opin Genet Dev *8*, 481-486.

Holinstat, M., Mehta, D., Kozasa, T., Minshall, R.D., and Malik, A.B. (2003). Protein kinase Calpha-induced p115RhoGEF phosphorylation signals endothelial cytoskeletal rearrangement. J Biol Chem *278*, 28793-28798.

Hollenberg, M.D., and Compton, S.J. (2002). International Union of Pharmacology. XXVIII. Proteinase-activated receptors. Pharmacol Rev *54*, 203-217.

Hollenberg, M.D., Saifeddine, M., al-Ani, B., and Kawabata, A. (1997). Proteinase-activated receptors: structural requirements for activity, receptor cross-reactivity, and receptor selectivity of receptor-activating peptides. Can J Physiol Pharmacol *75*, 832-841.

Hopkins, A.L., and Groom, C.R. (2002). The druggable genome. Nat Rev Drug Discov *1*, 727-730.

Horowitz, J.C., and Thannickal, V.J. (2006). Idiopathic pulmonary fibrosis : new concepts in pathogenesis and implications for drug therapy. Treat Respir Med *5*, 325-342.

Horvat, R., and Palade, G.E. (1995). The functional thrombin receptor is associated with the plasmalemma and a large endosomal network in cultured human umbilical vein endothelial cells. J Cell Sci *108 (Pt 3)*, 1155-1164.

Houliston, R.A., Keogh, R.J., Sugden, D., Dudhia, J., Carter, T.D., and Wheeler-Jones, C.P. (2002). Protease-activated receptors upregulate cyclooxygenase-2 expression in human endothelial cells. Thromb Haemost *88*, 321-328.

Howell, D.C., Johns, R.H., Lasky, J.A., Shan, B., Scotton, C.J., Laurent, G.J., and Chambers, R.C. (2005). Absence of proteinase-activated receptor-1 signaling affords protection from bleomycin-induced lung inflammation and fibrosis. Am J Pathol *166*, 1353-1365.

Howell, D.C., Laurent, G.J., and Chambers, R.C. (2002). Role of thrombin and its major cellular receptor, protease-activated receptor-1, in pulmonary fibrosis. Biochem Soc Trans *30*, 211-216.

Hoxie, J.A., Ahuja, M., Belmonte, E., Pizarro, S., Parton, R., and Brass, L.F. (1993). Internalization and recycling of activated thrombin receptors. J Biol Chem *268*, 13756-13763.

Hoyt, D.G., and Lazo, J.S. (1988). Alterations in pulmonary mRNA encoding procollagens, fibronectin and transforming growth factor-beta precede bleomycin-induced pulmonary fibrosis in mice. J Pharmacol Exp Ther *246*, 765-771.

Huang, L., Ogushi, F., Tani, K., Ogawa, H., Kawano, T., Endo, T., Izumi, K., Sono, N., Ueno, J., Nishitani, H., and Sone, S. (2001). Thrombin promotes fibroblast proliferation during the early stages of experimental radiation pneumonitis. Radiat Res *156*, 45-52.

Hubbard, K.B., and Hepler, J.R. (2006). Cell signalling diversity of the Gqalpha family of heterotrimeric G proteins. Cell Signal *18*, 135-150.

Hubbard, R., Johnston, I., and Britton, J. (1998). Survival in patients with cryptogenic fibrosing alveolitis: a population-based cohort study. Chest *113*, 396-400.

Hung, D.T., Vu, T.H., Nelken, N.A., and Coughlin, S.R. (1992a). Thrombin-induced events in non-platelet cells are mediated by the unique proteolytic mechanism established for the cloned platelet thrombin receptor. J Cell Biol *116*, 827-832.

Hung, D.T., Wong, Y.H., Vu, T.K., and Coughlin, S.R. (1992b). The cloned platelet thrombin receptor couples to at least two distinct effectors to stimulate phosphoinositide hydrolysis and inhibit adenylyl cyclase. J Biol Chem *267*, 20831-20834.

Idell, S. (2003). Coagulation, fibrinolysis, and fibrin deposition in acute lung injury. Crit Care Med *31*, S213-220.

Idell, S., James, K.K., Levin, E.G., Schwartz, B.S., Manchanda, N., Maunder, R.J., Martin, T.R., McLarty, J., and Fair, D.S. (1989). Local abnormalities in coagulation and fibrinolytic pathways predispose to alveolar fibrin deposition in the adult respiratory distress syndrome. J Clin Invest *84*, 695-705.

Ihnatko, R., and Kubes, M. (2007). TNF signaling: early events and phosphorylation. Gen Physiol Biophys *26*, 159-167.

Imokawa, S., Sato, A., Hayakawa, H., Kotani, M., Urano, T., and Takada, A. (1997). Tissue factor expression and fibrin deposition in the lungs of patients with idiopathic pulmonary fibrosis and systemic sclerosis. Am J Respir Crit Care Med *156*, 631-636.

Inoshima, I., Kuwano, K., Hamada, N., Hagimoto, N., Yoshimi, M., Maeyama, T., Takeshita, A., Kitamoto, S., Egashira, K., and Hara, N. (2004). Anti-monocyte chemoattractant protein-1 gene therapy attenuates pulmonary fibrosis in mice. Am J Physiol Lung Cell Mol Physiol *286*, L1038-1044.

Ip, W.K., Wong, C.K., Li, M.L., Li, P.W., Cheung, P.F., and Lam, C.W. (2007). Interleukin-31 induces cytokine and chemokine production from human bronchial epithelial cells through activation of mitogen-activated protein kinase signalling pathways: implications for the allergic response. Immunology *122*, 532-541.

Ishihara, H., Connolly, A.J., Zeng, D., Kahn, M.L., Zheng, Y.W., Timmons, C., Tram, T., and Coughlin, S.R. (1997). Protease-activated receptor 3 is a second thrombin receptor in humans. Nature *386*, 502-506.

Ishii, K., Chen, J., Ishii, M., Koch, W.J., Freedman, N.J., Lefkowitz, R.J., and Coughlin, S.R. (1994). Inhibition of thrombin receptor signaling by a G-protein coupled receptor kinase. Functional specificity among G-protein coupled receptor kinases. J Biol Chem *269*, 1125-1130.

Jantzen, H.M., Milstone, D.S., Gousset, L., Conley, P.B., and Mortensen, R.M. (2001). Impaired activation of murine platelets lacking G alpha(i2). J Clin Invest *108*, 477-483.

Jenkins, R.G., Su, X., Su, G., Scotton, C.J., Camerer, E., Laurent, G.J., Davis, G.E., Chambers, R.C., Matthay, M.A., and Sheppard, D. (2006). Ligation of protease-activated receptor 1 enhances alpha(v)beta6 integrin-dependent TGF-beta activation and promotes acute lung injury. J Clin Invest *116*, 1606-1614.

Jeong, S.K., Kim, H.J., Youm, J.K., Ahn, S.K., Choi, E.H., Sohn, M.H., Kim, K.E., Hong, J.H., Shin, D.M., and Lee, S.H. (2008). Mite and Cockroach Allergens Activate Protease-Activated Receptor 2 and Delay Epidermal Permeability Barrier Recovery. J Invest Dermatol.

John, J., Rensland, H., Schlichting, I., Vetter, I., Borasio, G.D., Goody, R.S., and Wittinghofer, A. (1993). Kinetic and structural analysis of the Mg(2+)-binding site of the guanine nucleotide-binding protein p21H-ras. J Biol Chem *268*, 923-929.

Johnson, G.L., and Lapadat, R. (2002). Mitogen-activated protein kinase pathways mediated by ERK, JNK, and p38 protein kinases. Science *298*, 1911-1912.

Johnson, K., Choi, Y., DeGroot, E., Samuels, I., Creasey, A., and Aarden, L. (1998). Potential mechanisms for a proinflammatory vascular cytokine response to coagulation activation. J Immunol *160*, 5130-5135.

Jordana, M., Sarnstrand, B., Sime, P.J., and Ramis, I. (1994). Immune-inflammatory functions of fibroblasts. Eur Respir J *7*, 2212-2222.

Jun, J.B., Kuechle, M., Harlan, J.M., and Elkon, K.B. (2003). Fibroblast and endothelial apoptosis in systemic sclerosis. Curr Opin Rheumatol *15*, 756-760.

Junge, C.E., Sugawara, T., Mannaioni, G., Alagarsamy, S., Conn, P.J., Brat, D.J., Chan, P.H., and Traynelis, S.F. (2003). The contribution of protease-activated receptor

1 to neuronal damage caused by transient focal cerebral ischemia. Proc Natl Acad Sci U S A *100*, 13019-13024.

Kahn, M.L., Nakanishi-Matsui, M., Shapiro, M.J., Ishihara, H., and Coughlin, S.R. (1999). Protease-activated receptors 1 and 4 mediate activation of human platelets by thrombin. J Clin Invest *103*, 879-887.

Kahn, M.L., Zheng, Y.W., Huang, W., Bigornia, V., Zeng, D., Moff, S., Farese, R.V., Jr., Tam, C., and Coughlin, S.R. (1998). A dual thrombin receptor system for platelet activation. Nature *394*, 690-694.

Kaibuchi, K., Kuroda, S., and Amano, M. (1999). Regulation of the cytoskeleton and cell adhesion by the Rho family GTPases in mammalian cells. Annu Rev Biochem *68*, 459-486.

Kalluri, R., and Neilson, E.G. (2003). Epithelial-mesenchymal transition and its implications for fibrosis. J Clin Invest *112*, 1776-1784.

Kaneider, N.C., Leger, A.J., Agarwal, A., Nguyen, N., Perides, G., Derian, C., Covic, L., and Kuliopulos, A. (2007). 'Role reversal' for the receptor PAR1 in sepsis-induced vascular damage. Nat Immunol *8*, 1303-1312.

Kaplanski, G., Fabrigoule, M., Boulay, V., Dinarello, C.A., Bongrand, P., Kaplanski, S., and Farnarier, C. (1997). Thrombin induces endothelial type II activation in vitro: IL-1 and TNF-alpha-independent IL-8 secretion and E-selectin expression. J Immunol *158*, 5435-5441.

Kaplanski, G., Marin, V., Fabrigoule, M., Boulay, V., Benoliel, A.M., Bongrand, P., Kaplanski, S., and Farnarier, C. (1998). Thrombin-activated human endothelial cells support monocyte adhesion in vitro following expression of intercellular adhesion molecule-1 (ICAM-1; CD54) and vascular cell adhesion molecule-1 (VCAM-1; CD106). Blood *92*, 1259-1267.

Karpus, W.J., Lukacs, N.W., Kennedy, K.J., Smith, W.S., Hurst, S.D., and Barrett, T.A. (1997). Differential CC chemokine-induced enhancement of T helper cell cytokine production. J Immunol *158*, 4129-4136.

Kasper, M., and Haroske, G. (1996). Alterations in the alveolar epithelium after injury leading to pulmonary fibrosis. Histol Histopathol *11*, 463-483.

Keane, M.P., Arenberg, D.A., Lynch, J.P., 3rd, Whyte, R.I., Iannettoni, M.D., Burdick, M.D., Wilke, C.A., Morris, S.B., Glass, M.C., DiGiovine, B., Kunkel, S.L., and Strieter, R.M. (1997). The CXC chemokines, IL-8 and IP-10, regulate angiogenic activity in idiopathic pulmonary fibrosis. J Immunol *159*, 1437-1443.

Keane, M.P., Strieter, R.M., and Belperio, J.A. (2005). Mechanisms and mediators of pulmonary fibrosis. Crit Rev Immunol *25*, 429-463.

Keane, M.P., Strieter, R.M., Lynch, J.P., 3rd, and Belperio, J.A. (2006). Inflammation and angiogenesis in fibrotic lung disease. Semin Respir Crit Care Med *27*, 589-599.

Kelly, M.M., Leigh, R., Gilpin, S.E., Cheng, E., Martin, G.E., Radford, K., Cox, G., and Gauldie, J. (2006). Cell-specific gene expression in patients with usual interstitial pneumonia. Am J Respir Crit Care Med *174*, 557-565.

Khalil, N., and Greenberg, A.H. (1991). The role of TGF-beta in pulmonary fibrosis. Ciba Found Symp *157*, 194-207; discussion 207-111.

Khalil, N., O'Connor, R., Unruh, H., Warren, P., Kemp, A., and Greenberg, A. (1991). Enhanced expression and immunohistochemical distribution of transforming growth factor-beta in idiopathic pulmonary fibrosis. Chest *99*, 65S-66S.

Khalil, N., O'Connor, R.N., Flanders, K.C., and Unruh, H. (1996). TGF-beta 1, but not TGF-beta 2 or TGF-beta 3, is differentially present in epithelial cells of advanced pulmonary fibrosis: an immunohistochemical study. Am J Respir Cell Mol Biol *14*, 131-138.

Kijiyama, N., Ueno, H., Sugimoto, I., Sasaguri, Y., Yatera, K., Kido, M., Gabazza, E.C., Suzuki, K., Hashimoto, E., and Takeya, H. (2006). Intratracheal gene transfer of tissue factor pathway inhibitor attenuates pulmonary fibrosis. Biochem Biophys Res Commun *339*, 1113-1119.

Kim, D.S., Collard, H.R., and King, T.E., Jr. (2006). Classification and natural history of the idiopathic interstitial pneumonias. Proc Am Thorac Soc *3*, 285-292.

Kimura, K., Ito, M., Amano, M., Chihara, K., Fukata, Y., Nakafuku, M., Yamamori, B., Feng, J., Nakano, T., Okawa, K., Iwamatsu, A., and Kaibuchi, K. (1996). Regulation of myosin phosphatase by Rho and Rho-associated kinase (Rho-kinase). Science *273*, 245-248.

Kinser, S., Jia, Q., Li, M., Laughter, A., Cornwell, P., Corton, J.C., and Pestka, J. (2004). Gene expression profiling in spleens of deoxynivalenol-exposed mice: immediate early genes as primary targets. J Toxicol Environ Health A *67*, 1423-1441.

Kobayashi, H., Gabazza, E.C., Taguchi, O., Wada, H., Takeya, H., Nishioka, J., Yasui, H., Kobayashi, T., Hataji, O., Suzuki, K., and Adachi, Y. (1998). Protein C anticoagulant system in patients with interstitial lung disease. Am J Respir Crit Care Med *157*, 1850-1854.

Kolb, M., Margetts, P.J., Anthony, D.C., Pitossi, F., and Gauldie, J. (2001). Transient expression of IL-1beta induces acute lung injury and chronic repair leading to pulmonary fibrosis. J Clin Invest *107*, 1529-1536.

Kolch, W. (2000). Meaningful relationships: the regulation of the Ras/Raf/MEK/ERK pathway by protein interactions. Biochem J *351 Pt 2*, 289-305.

Kolch, W., Kotwaliwale, A., Vass, K., and Janosch, P. (2002). The role of Raf kinases in malignant transformation. Expert Rev Mol Med *4*, 1-18.

Komarova, Y.A., Mehta, D., and Malik, A.B. (2007). Dual regulation of endothelial junctional permeability. Sci STKE *2007*, re8.

Kong, W., McConalogue, K., Khitin, L.M., Hollenberg, M.D., Payan, D.G., Bohm, S.K., and Bunnett, N.W. (1997). Luminal trypsin may regulate enterocytes through proteinase-activated receptor 2. Proc Natl Acad Sci U S A *94*, 8884-8889.

Konstan, M.W., and Davis, P.B. (2002). Pharmacological approaches for the discovery and development of new anti-inflammatory agents for the treatment of cystic fibrosis. Adv Drug Deliv Rev *54*, 1409-1423.

Kotani, I., Sato, A., Hayakawa, H., Urano, T., Takada, Y., and Takada, A. (1995). Increased procoagulant and antifibrinolytic activities in the lungs with idiopathic pulmonary fibrosis. Thromb Res *77*, 493-504.

Kozasa, T., Jiang, X., Hart, M.J., Sternweis, P.M., Singer, W.D., Gilman, A.G., Bollag, G., and Sternweis, P.C. (1998). p115 RhoGEF, a GTPase activating protein for Galpha12 and Galpha13. Science *280*, 2109-2111.

Kranzhofer, R., Clinton, S.K., Ishii, K., Coughlin, S.R., Fenton, J.W., 2nd, and Libby, P. (1996). Thrombin potently stimulates cytokine production in human vascular smooth muscle cells but not in mononuclear phagocytes. Circ Res *79*, 286-294.

Krymskaya, V.P., Penn, R.B., Orsini, M.J., Scott, P.H., Plevin, R.J., Walker, T.R., Eszterhas, A.J., Amrani, Y., Chilvers, E.R., and Panettieri, R.A., Jr. (1999). Phosphatidylinositol 3-kinase mediates mitogen-induced human airway smooth muscle cell proliferation. Am J Physiol *277*, L65-78.

Kubo, H., Nakayama, K., Yanai, M., Suzuki, T., Yamaya, M., Watanabe, M., and Sasaki, H. (2005). Anticoagulant therapy for idiopathic pulmonary fibrosis. Chest *128*, 1475-1482.

Kuo, W.H., Chen, J.H., Lin, H.H., Chen, B.C., Hsu, J.D., and Wang, C.J. (2005). Induction of apoptosis in the lung tissue from rats exposed to cigarette smoke involves p38/JNK MAPK pathway. Chem Biol Interact *155*, 31-42.

Kuwano, K. (2007). Epithelial cell apoptosis and lung remodeling. Cell Mol Immunol *4*, 419-429.

Kyriakis, J.M., and Avruch, J. (2001). Mammalian mitogen-activated protein kinase signal transduction pathways activated by stress and inflammation. Physiol Rev *81*, 807-869.

LaMorte, V.J., Harootunian, A.T., Spiegel, A.M., Tsien, R.Y., and Feramisco, J.R. (1993). Mediation of growth factor induced DNA synthesis and calcium mobilization by Gq and Gi2. J Cell Biol *121*, 91-99.

Lasky, J.A., Ortiz, L.A., Tonthat, B., Hoyle, G.W., Corti, M., Athas, G., Lungarella, G., Brody, A., and Friedman, M. (1998). Connective tissue growth factor mRNA expression is upregulated in bleomycin-induced lung fibrosis. Am J Physiol *275*, L365-371.

Laurent, G., Shapiro, S. (2007). Encyclopedia of Respiratory Medicine. Academic Press.

Lawrence, T., Gilroy, D.W., Colville-Nash, P.R., and Willoughby, D.A. (2001). Possible new role for NF-kappaB in the resolution of inflammation. Nat Med *7*, 1291-1297.

Lawson, W.E., Grant, S.W., Ambrosini, V., Womble, K.E., Dawson, E.P., Lane, K.B., Markin, C., Renzoni, E., Lympany, P., Thomas, A.Q., Roldan, J., Scott, T.A., Blackwell, T.S., Phillips, J.A., 3rd, Loyd, J.E., and du Bois, R.M. (2004). Genetic mutations in surfactant protein C are a rare cause of sporadic cases of IPF. Thorax *59*, 977-980.

Leask, A., and Abraham, D.J. (2003). The role of connective tissue growth factor, a multifunctional matricellular protein, in fibroblast biology. Biochem Cell Biol *81*, 355-363.

Lee, H.R., Ho, W.Z., and Douglas, S.D. (1994). Substance P augments tumor necrosis factor release in human monocyte-derived macrophages. Clin Diagn Lab Immunol *1*, 419-423.

Lee, J.H., Johnson, P.R., Roth, M., Hunt, N.H., and Black, J.L. (2001). ERK activation and mitogenesis in human airway smooth muscle cells. Am J Physiol Lung Cell Mol Physiol *280*, L1019-1029.

Leger, A.J., Covic, L., and Kuliopulos, A. (2006). Protease-activated receptors in cardiovascular diseases. Circulation *114*, 1070-1077.

Lepailleur-Enouf, D., Valdenaire, O., Philippe, M., Jandrot-Perrus, M., and Michel, J.B. (2000). Thrombin induces endothelin expression in arterial smooth muscle cells. Am J Physiol Heart Circ Physiol *278*, H1606-1612.

Leslie, C.C., and Detty, D.M. (1986). Arachidonic acid turnover in response to lipopolysaccharide and opsonized zymosan in human monocyte-derived macrophages. Biochem J *236*, 251-259.

Li, X., Hahn, C.N., Parsons, M., Drew, J., Vadas, M.A., and Gamble, J.R. (2004). Role of protein kinase Czeta in thrombin-induced endothelial permeability changes: inhibition by angiopoietin-1. Blood *104*, 1716-1724.

Lidington, E.A., Steinberg, R., Kinderlerer, A.R., Landis, R.C., Ohba, M., Samarel, A., Haskard, D.O., and Mason, J.C. (2005). A role for proteinase-activated receptor 2 and PKC-epsilon in thrombin-mediated induction of decay-accelerating factor on human endothelial cells. Am J Physiol Cell Physiol *289*, C1437-1447.

Lin, A. (2003). Activation of the JNK signaling pathway: breaking the brake on apoptosis. Bioessays *25*, 17-24.

Lin, C.C., Shyr, M.H., Chien, C.S., Wang, C.C., Chiu, C.T., Hsiao, L.D., and Yang, C.M. (2002). Thrombin-stimulated cell proliferation mediated through activation of Ras/Raf/MEK/MAPK pathway in canine cultured tracheal smooth muscle cells. Cell Signal *14*, 265-275.

Littler, C.M., Morris, K.G., Jr., Fagan, K.A., McMurtry, I.F., Messing, R.O., and Dempsey, E.C. (2003). Protein kinase C-epsilon-null mice have decreased hypoxic pulmonary vasoconstriction. Am J Physiol Heart Circ Physiol *284*, H1321-1331.

Littler, C.M., Wehling, C.A., Wick, M.J., Fagan, K.A., Cool, C.D., Messing, R.O., and Dempsey, E.C. (2005). Divergent contractile and structural responses of the murine PKC-epsilon null pulmonary circulation to chronic hypoxia. Am J Physiol Lung Cell Mol Physiol *289*, L1083-1093.

Liu, J., and Lin, A. (2005). Role of JNK activation in apoptosis: a double-edged sword. Cell Res *15*, 36-42.

Liu, X., Das, A.M., Seideman, J., Griswold, D., Afuh, C.N., Kobayashi, T., Abe, S., Fang, Q., Hashimoto, M., Kim, H., Wang, X., Shen, L., Kawasaki, S., and Rennard, S.I. (2007). The CC chemokine ligand 2 (CCL2) mediates fibroblast survival through IL-6. Am J Respir Cell Mol Biol *37*, 121-128.

Lo, S.K., Perlman, M.B., Niehaus, G.D., and Malik, A.B. (1985). Thrombin-induced alterations in lung fluid balance in awake sheep. J Appl Physiol *58*, 1421-1427.

Lu, B., Rutledge, B.J., Gu, L., Fiorillo, J., Lukacs, N.W., Kunkel, S.L., North, R., Gerard, C., and Rollins, B.J. (1998). Abnormalities in monocyte recruitment and cytokine expression in monocyte chemoattractant protein 1-deficient mice. J Exp Med *187*, 601-608.

Ludwicka-Bradley, A., Tourkina, E., Suzuki, S., Tyson, E., Bonner, M., Fenton, J.W., 2nd, Hoffman, S., and Silver, R.M. (2000). Thrombin upregulates interleukin-8 in lung fibroblasts via cleavage of proteolytically activated receptor-I and protein kinase C-gamma activation. Am J Respir Cell Mol Biol *22*, 235-243.

Ludwicka, A., Trojanowska, M., Smith, E.A., Baumann, M., Strange, C., Korn, J.H., Smith, T., Leroy, E.C., and Silver, R.M. (1992). Growth and characterization of fibroblasts obtained from bronchoalveolar lavage of patients with scleroderma. J Rheumatol *19*, 1716-1723.

Lum, H., Andersen, T.T., Siflinger-Birnboim, A., Tiruppathi, C., Goligorsky, M.S., Fenton, J.W., 2nd, and Malik, A.B. (1993). Thrombin receptor peptide inhibits thrombin-induced increase in endothelial permeability by receptor desensitization. J Cell Biol *120*, 1491-1499.

Luna, M.A., Bedrossian, C.W., Lichtiger, B., and Salem, P.A. (1972). Interstitial pneumonitis associated with bleomycin therapy. Am J Clin Pathol *58*, 501-510.

Lutz, S., Freichel-Blomquist, A., Yang, Y., Rumenapp, U., Jakobs, K.H., Schmidt, M., and Wieland, T. (2005). The guanine nucleotide exchange factor p63RhoGEF, a specific link between Gq/11-coupled receptor signaling and RhoA. J Biol Chem *280*, 11134-11139.

Lutz, S., Shankaranarayanan, A., Coco, C., Ridilla, M., Nance, M.R., Vettel, C., Baltus, D., Evelyn, C.R., Neubig, R.R., Wieland, T., and Tesmer, J.J. (2007). Structure of Galphaq-p63RhoGEF-RhoA complex reveals a pathway for the activation of RhoA by GPCRs. Science *318*, 1923-1927.

Luzina, I.G., Highsmith, K., Pochetuhen, K., Nacu, N., Rao, J.N., and Atamas, S.P. (2006). PKCalpha mediates CCL18-stimulated collagen production in pulmonary fibroblasts. Am J Respir Cell Mol Biol *35*, 298-305.

Lynch, J.P., 3rd, Standiford, T.J., Rolfe, M.W., Kunkel, S.L., and Strieter, R.M. (1992). Neutrophilic alveolitis in idiopathic pulmonary fibrosis. The role of interleukin-8. Am Rev Respir Dis *145*, 1433-1439.

Macfarlane, S.R., Seatter, M.J., Kanke, T., Hunter, G.D., and Plevin, R. (2001). Proteinase-activated receptors. Pharmacol Rev *53*, 245-282.

Maher, T.M., Wells, A.U., and Laurent, G.J. (2007). Idiopathic pulmonary fibrosis: multiple causes and multiple mechanisms? Eur Respir J *30*, 835-839.

Major, C.D., Santulli, R.J., Derian, C.K., and Andrade-Gordon, P. (2003). Extracellular mediators in atherosclerosis and thrombosis: lessons from thrombin receptor knockout mice. Arterioscler Thromb Vasc Biol *23*, 931-939.

Mandal, S.K., Rao, L.V., Tran, T.T., and Pendurthi, U.R. (2005). A novel mechanism of plasmin-induced mitogenesis in fibroblasts. J Thromb Haemost *3*, 163-169.

Mapel, D.W., Hunt, W.C., Utton, R., Baumgartner, K.B., Samet, J.M., and Coultas, D.B. (1998). Idiopathic pulmonary fibrosis: survival in population based and hospital based cohorts. Thorax *53*, 469-476.

Marin, V., Farnarier, C., Gres, S., Kaplanski, S., Su, M.S., Dinarello, C.A., and Kaplanski, G. (2001). The p38 mitogen-activated protein kinase pathway plays a critical role in thrombin-induced endothelial chemokine production and leukocyte recruitment. Blood *98*, 667-673.

Marinissen, M.J., Servitja, J.M., Offermanns, S., Simon, M.I., and Gutkind, J.S. (2003). Thrombin protease-activated receptor-1 signals through Gq- and G13-initiated MAPK cascades regulating c-Jun expression to induce cell transformation. J Biol Chem *278*, 46814-46825.

Markowitz, D., Goff, S., and Bank, A. (1988). Construction of a safe and efficient retrovirus packaging cell line. Adv Exp Med Biol *241*, 35-40.
Marsen, T.A., Simonson, M.S., and Dunn, M.J. (1995). Thrombin-mediated ET-1 gene regulation involves CaM kinases and calcineurin in human endothelial cells. J Cardiovasc Pharmacol *26 Suppl 3*, S1-4.

Martin, C.B., Mahon, G.M., Klinger, M.B., Kay, R.J., Symons, M., Der, C.J., and Whitehead, I.P. (2001). The thrombin receptor, PAR-1, causes transformation by activation of Rho-mediated signaling pathways. Oncogene *20*, 1953-1963.

Martiny-Baron, G., Kazanietz, M.G., Mischak, H., Blumberg, P.M., Kochs, G., Hug, H., Marme, D., and Schachtele, C. (1993). Selective inhibition of protein kinase C isozymes by the indolocarbazole Go 6976. J Biol Chem *268*, 9194-9197.

Maruyama, N., Hirano, F., Yoshikawa, N., Migita, K., Eguchi, K., and Tanaka, H. (2000). Thrombin stimulates cell proliferation in human fibroblast-like synoviocytes in nuclear factor-kappaB activation and protein kinase C mediated pathway. J Rheumatol *27*, 2777-2785.

Maryanoff, B.E., Zhang, H.C., Andrade-Gordon, P., and Derian, C.K. (2003). Discovery of potent peptide-mimetic antagonists for the human thrombin receptor, protease-activated receptor-1 (PAR-1). Curr Med Chem Cardiovasc Hematol Agents *1*, 13-36.

McLaughlin, J.N., Patterson, M.M., and Malik, A.B. (2007). Protease-activated receptor-3 (PAR3) regulates PAR1 signaling by receptor dimerization. Proc Natl Acad Sci U S A *104*, 5662-5667.

McLaughlin, J.N., Shen, L., Holinstat, M., Brooks, J.D., Dibenedetto, E., and Hamm, H.E. (2005). Functional selectivity of G protein signaling by agonist peptides and thrombin for the protease-activated receptor-1. J Biol Chem *280*, 25048-25059.

Mehrad, B., Burdick, M.D., Zisman, D.A., Keane, M.P., Belperio, J.A., and Strieter, R.M. (2007). Circulating peripheral blood fibrocytes in human fibrotic interstitial lung disease. Biochem Biophys Res Commun *353*, 104-108.

Mehta, D., and Malik, A.B. (2006). Signaling mechanisms regulating endothelial permeability. Physiol Rev *86*, 279-367.

Miho, N., Ishida, T., Kuwaba, N., Ishida, M., Shimote-Abe, K., Tabuchi, K., Oshima, T., Yoshizumi, M., and Chayama, K. (2005). Role of the JNK pathway in thrombin-induced ICAM-1 expression in endothelial cells. Cardiovasc Res *68*, 289-298.

Miller, E.J., Cohen, A.B., Nagao, S., Griffith, D., Maunder, R.J., Martin, T.R., Weiner-Kronish, J.P., Sticherling, M., Christophers, E., and Matthay, M.A. (1992). Elevated levels of NAP-1/interleukin-8 are present in the airspaces of patients with the adult respiratory distress syndrome and are associated with increased mortality. Am Rev Respir Dis *146*, 427-432.

Milligan, G. (2001). Oligomerisation of G-protein-coupled receptors. J Cell Sci *114*, 1265-1271.

Missy, K., Plantavid, M., Pacaud, P., Viala, C., Chap, H., and Payrastre, B. (2001). Rho-kinase is involved in the sustained phosphorylation of myosin and the irreversible platelet aggregation induced by PAR1 activating peptide. Thromb Haemost *85*, 514-520.

Mochly-Rosen, D., and Gordon, A.S. (1998). Anchoring proteins for protein kinase C: a means for isozyme selectivity. FASEB J *12*, 35-42.

Moers, A., Wettschureck, N., Gruner, S., Nieswandt, B., and Offermanns, S. (2004). Unresponsiveness of platelets lacking both Galpha(q) and Galpha(13). Implications for collagen-induced platelet activation. J Biol Chem *279*, 45354-45359.

Moodley, Y.P., Caterina, P., Scaffidi, A.K., Misso, N.L., Papadimitriou, J.M., McAnulty, R.J., Laurent, G.J., Thompson, P.J., and Knight, D.A. (2004). Comparison of the morphological and biochemical changes in normal human lung fibroblasts and fibroblasts derived from lungs of patients with idiopathic pulmonary fibrosis during FasL-induced apoptosis. J Pathol *202*, 486-495.

Moore, B.B., Kolodsick, J.E., Thannickal, V.J., Cooke, K., Moore, T.A., Hogaboam, C., Wilke, C.A., and Toews, G.B. (2005). CCR2-mediated recruitment of fibrocytes to the alveolar space after fibrotic injury. Am J Pathol *166*, 675-684.

Moore, B.B., Murray, L., Das, A., Wilke, C.A., Herrygers, A.B., and Toews, G.B. (2006). The role of CCL12 in the recruitment of fibrocytes and lung fibrosis. Am J Respir Cell Mol Biol *35*, 175-181.

Moore, B.B., Paine, R., 3rd, Christensen, P.J., Moore, T.A., Sitterding, S., Ngan, R., Wilke, C.A., Kuziel, W.A., and Toews, G.B. (2001). Protection from pulmonary fibrosis in the absence of CCR2 signaling. J Immunol *167*, 4368-4377.

Moore, B.B., Peters-Golden, M., Christensen, P.J., Lama, V., Kuziel, W.A., Paine, R., 3rd, and Toews, G.B. (2003). Alveolar epithelial cell inhibition of fibroblast proliferation is regulated by MCP-1/CCR2 and mediated by PGE2. Am J Physiol Lung Cell Mol Physiol *284*, L342-349.

Murray, L.A., Argentieri, R.L., Farrell, F.X., Bracht, M., Sheng, H., Whitaker, B., Beck, H., Tsui, P., Cochlin, K., Evanoff, H.L., Hogaboam, C.M., and Das, A.M. (2008). Hyper-responsiveness of IPF/UIP fibroblasts: Interplay between TGFbeta1, IL-13 and CCL2. Int J Biochem Cell Biol.

Nakanishi-Matsui, M., Zheng, Y.W., Sulciner, D.J., Weiss, E.J., Ludeman, M.J., and Coughlin, S.R. (2000). PAR3 is a cofactor for PAR4 activation by thrombin. Nature *404*, 609-613.

Naldini, A., Carney, D.H., Pucci, A., Pasquali, A., and Carraro, F. (2000). Thrombin regulates the expression of proangiogenic cytokines via proteolytic activation of protease-activated receptor-1. Gen Pharmacol *35*, 255-259.

Naldini, A., Pucci, A., Carney, D.H., Fanetti, G., and Carraro, F. (2002). Thrombin enhancement of interleukin-1 expression in mononuclear cells: involvement of proteinase-activated receptor-1. Cytokine *20*, 191-199.

Nguyen, Q.D., De Wever, O., Bruyneel, E., Hendrix, A., Xie, W.Z., Lombet, A., Leibl, M., Mareel, M., Gieseler, F., Bracke, M., and Gespach, C. (2005). Commutators of PAR-1 signaling in cancer cell invasion reveal an essential role of the Rho-Rho kinase axis and tumor microenvironment. Oncogene *24*, 8240-8251.

Nocker, R.E., Schoonbrood, D.F., van de Graaf, E.A., Hack, C.E., Lutter, R., Jansen, H.M., and Out, T.A. (1996). Interleukin-8 in airway inflammation in patients with asthma and chronic obstructive pulmonary disease. Int Arch Allergy Immunol *109*, 183-191.
O'Brien, P.J., Molino, M., Kahn, M., and Brass, L.F. (2001). Protease activated receptors: theme and variations. Oncogene *20*, 1570-1581.

O'Brien, P.J., Prevost, N., Molino, M., Hollinger, M.K., Woolkalis, M.J., Woulfe, D.S., and Brass, L.F. (2000). Thrombin responses in human endothelial cells. Contributions from receptors other than PAR1 include the transactivation of PAR2 by thrombin-cleaved PAR1. J Biol Chem *275*, 13502-13509.

Offermanns, S., Laugwitz, K.L., Spicher, K., and Schultz, G. (1994). G proteins of the G12 family are activated via thromboxane A2 and thrombin receptors in human platelets. Proc Natl Acad Sci U S A *91*, 504-508.

Offermanns, S., Toombs, C.F., Hu, Y.H., and Simon, M.I. (1997). Defective platelet activation in G alpha(q)-deficient mice. Nature *389*, 183-186.

Ohba, T., McDonald, J.K., Silver, R.M., Strange, C., LeRoy, E.C., and Ludwicka, A. (1994). Scleroderma bronchoalveolar lavage fluid contains thrombin, a mediator of human lung fibroblast proliferation via induction of platelet-derived growth factor alpha-receptor. Am J Respir Cell Mol Biol *10*, 405-412.

Oriente, A., Fedarko, N.S., Pacocha, S.E., Huang, S.K., Lichtenstein, L.M., and Essayan, D.M. (2000). Interleukin-13 modulates collagen homeostasis in human skin and keloid fibroblasts. J Pharmacol Exp Ther *292*, 988-994.

Ossovskaya, V.S., and Bunnett, N.W. (2004). Protease-activated receptors: contribution to physiology and disease. Physiol Rev *84*, 579-621.

Ostrowska, E., and Reiser, G. (2008a). The protease-activated receptor-3 (PAR-3) can signal autonomously to induce interleukin-8 release. Cell Mol Life Sci *65*, 970-981.

Ostrowska, E., and Reiser, G. (2008b). Protease-activated receptor (PAR)-induced interleukin-8 production in airway epithelial cells requires activation of MAP kinases p44/42 and JNK. Biochem Biophys Res Commun *366*, 1030-1035.

Pages, G., Lenormand, P., L'Allemain, G., Chambard, J.C., Meloche, S., and Pouyssegur, J. (1993). Mitogen-activated protein kinases p42mapk and p44mapk are required for fibroblast proliferation. Proc Natl Acad Sci U S A *90*, 8319-8323.

Pan, T., Mason, R.J., Westcott, J.Y., and Shannon, J.M. (2001). Rat alveolar type II cells inhibit lung fibroblast proliferation in vitro. Am J Respir Cell Mol Biol *25*, 353-361.

Papiris, S.A., Kollintza, A., Kitsanta, P., Kapotsis, G., Karatza, M., Milic-Emili, J., Roussos, C., and Daniil, Z. (2005). Relationship of BAL and lung tissue CD4+ and CD8+ T lymphocytes, and their ratio in idiopathic pulmonary fibrosis. Chest *128*, 2971-2977.

Parambil, J.G., Myers, J.L., and Ryu, J.H. (2005). Histopathologic features and outcome of patients with acute exacerbation of idiopathic pulmonary fibrosis undergoing surgical lung biopsy. Chest *128*, 3310-3315.

Parekh, D.B., Ziegler, W., and Parker, P.J. (2000). Multiple pathways control protein kinase C phosphorylation. EMBO J *19*, 496-503.

Pawlinski, R., Pedersen, B., Schabbauer, G., Tencati, M., Holscher, T., Boisvert, W., Andrade-Gordon, P., Frank, R.D., and Mackman, N. (2004). Role of tissue factor and protease-activated receptors in a mouse model of endotoxemia. Blood *103*, 1342-1347.

Pelaia, G., Cuda, G., Vatrella, A., Gallelli, L., Caraglia, M., Marra, M., Abbruzzese, A., Caputi, M., Maselli, R., Costanzo, F.S., and Marsico, S.A. (2005). Mitogen-activated protein kinases and asthma. J Cell Physiol *202*, 642-653.

Petkova, D.K., Clelland, C.A., Ronan, J.E., Lewis, S., and Knox, A.J. (2003). Reduced expression of cyclooxygenase (COX) in idiopathic pulmonary fibrosis and sarcoidosis. Histopathology *43*, 381-386.

Peyrol, S., Cordier, J.F., and Grimaud, J.A. (1990). Intra-alveolar fibrosis of idiopathic bronchiolitis obliterans-organizing pneumonia. Cell-matrix patterns. Am J Pathol *137*, 155-170.

Phan, S.H. (2002). The myofibroblast in pulmonary fibrosis. Chest *122*, 286S-289S.

Phillips, R.J., Burdick, M.D., Hong, K., Lutz, M.A., Murray, L.A., Xue, Y.Y., Belperio, J.A., Keane, M.P., and Strieter, R.M. (2004). Circulating fibrocytes traffic to the lungs in response to CXCL12 and mediate fibrosis. J Clin Invest *114*, 438-446.

Piguet, P.F., Van, G.Y., and Guo, J. (1996). Heparin attenuates bleomycin but not silica-induced pulmonary fibrosis in mice: possible relationship with involvement of myofibroblasts in bleomycin, and fibroblasts in silica-induced fibrosis. Int J Exp Pathol *77*, 155-161.

Piguet, P.F., and Vesin, C. (1994). Pulmonary platelet trapping induced by bleomycin: correlation with fibrosis and involvement of the beta 2 integrins. Int J Exp Pathol *75*, 321-328.

Ping, D., Boekhoudt, G., and Boss, J.M. (1999a). trans-Retinoic acid blocks platelet-derived growth factor-BB-induced expression of the murine monocyte chemoattractant-1 gene by blocking the assembly of a promoter proximal Sp1 binding site. J Biol Chem *274*, 31909-31916.

Ping, D., Boekhoudt, G., Zhang, F., Morris, A., Philipsen, S., Warren, S.T., and Boss, J.M. (2000). Sp1 binding is critical for promoter assembly and activation of the MCP-1 gene by tumor necrosis factor. J Biol Chem *275*, 1708-1714.

Ping, D., Boekhoudt, G.H., Rogers, E.M., and Boss, J.M. (1999b). Nuclear factor-kappa B p65 mediates the assembly and activation of the TNF-responsive element of the murine monocyte chemoattractant-1 gene. J Immunol *162*, 727-734.

Ping, D., Jones, P.L., and Boss, J.M. (1996). TNF regulates the in vivo occupancy of both distal and proximal regulatory regions of the MCP-1/JE gene. Immunity *4*, 455-469.

Plataki, M., Koutsopoulos, A.V., Darivianaki, K., Delides, G., Siafakas, N.M., and Bouros, D. (2005). Expression of apoptotic and antiapoptotic markers in epithelial cells in idiopathic pulmonary fibrosis. Chest *127*, 266-274.

Puneet, P., Moochhala, S., and Bhatia, M. (2005). Chemokines in acute respiratory distress syndrome. Am J Physiol Lung Cell Mol Physiol *288*, L3-15.

Rabiet, M.J., Plantier, J.L., Rival, Y., Genoux, Y., Lampugnani, M.G., and Dejana, E. (1996). Thrombin-induced increase in endothelial permeability is associated with changes in cell-to-cell junction organization. Arterioscler Thromb Vasc Biol *16*, 488-496.

Raghu, G. (2006). Idiopathic pulmonary fibrosis: treatment options in pursuit of evidence-based approaches. Eur Respir J *28*, 463-465.

Raghu, G., Chen, Y.Y., Rusch, V., and Rabinovitch, P.S. (1988). Differential proliferation of fibroblasts cultured from normal and fibrotic human lungs. Am Rev Respir Dis *138*, 703-708.

Rahman, A., Anwar, K.N., True, A.L., and Malik, A.B. (1999). Thrombin-induced p65 homodimer binding to downstream NF-kappa B site of the promoter mediates endothelial ICAM-1 expression and neutrophil adhesion. J Immunol *162*, 5466-5476.

Rahman, A., Anwar, K.N., Uddin, S., Xu, N., Ye, R.D., Platanias, L.C., and Malik, A.B. (2001). Protein kinase C-delta regulates thrombin-induced ICAM-1 gene expression in endothelial cells via activation of p38 mitogen-activated protein kinase. Mol Cell Biol *21*, 5554-5565.

Rahman, A., True, A.L., Anwar, K.N., Ye, R.D., Voyno-Yasenetskaya, T.A., and Malik, A.B. (2002). Galpha(q) and Gbetagamma regulate PAR-1 signaling of thrombin-induced NF-kappaB activation and ICAM-1 transcription in endothelial cells. Circ Res *91*, 398-405.

Rahman, I., and MacNee, W. (1998). Role of transcription factors in inflammatory lung diseases. Thorax *53*, 601-612.

Ramos, C., Montano, M., Garcia-Alvarez, J., Ruiz, V., Uhal, B.D., Selman, M., and Pardo, A. (2001). Fibroblasts from idiopathic pulmonary fibrosis and normal lungs differ in growth rate, apoptosis, and tissue inhibitor of metalloproteinases expression. Am J Respir Cell Mol Biol *24*, 591-598.

Renesto, P., Si-Tahar, M., Moniatte, M., Balloy, V., Van Dorsselaer, A., Pidard, D., and Chignard, M. (1997). Specific inhibition of thrombin-induced cell activation by the neutrophil proteinases elastase, cathepsin G, and proteinase 3: evidence for distinct cleavage sites within the aminoterminal domain of the thrombin receptor. Blood *89*, 1944-1953.

Riewald, M., Kravchenko, V.V., Petrovan, R.J., O'Brien, P.J., Brass, L.F., Ulevitch, R.J., and Ruf, W. (2001). Gene induction by coagulation factor Xa is mediated by activation of protease-activated receptor 1. Blood *97*, 3109-3116.

Riewald, M., Petrovan, R.J., Donner, A., Mueller, B.M., and Ruf, W. (2002). Activation of endothelial cell protease activated receptor 1 by the protein C pathway. Science *296*, 1880-1882.

Riewald, M., and Ruf, W. (2005). Protease-activated receptor-1 signaling by activated protein C in cytokine-perturbed endothelial cells is distinct from thrombin signaling. J Biol Chem *280*, 19808-19814.

Rose, C.E., Jr., Sung, S.S., and Fu, S.M. (2003). Significant involvement of CCL2 (MCP-1) in inflammatory disorders of the lung. Microcirculation *10*, 273-288.

Roy, S.S., Saifeddine, M., Loutzenhiser, R., Triggle, C.R., and Hollenberg, M.D. (1998). Dual endothelium-dependent vascular activities of proteinase-activated receptor-2-activating peptides: evidence for receptor heterogeneity. Br J Pharmacol *123*, 1434-1440.

Sabatini, F., Silvestri, M., Sale, R., Scarso, L., Defilippi, A.C., Risso, F.M., and Rossi, G.A. (2002). Fibroblast-eosinophil interaction: modulation of adhesion molecules expression and chemokine release by human fetal lung fibroblasts in response to IL-4 and TNF-alpha. Immunol Lett *84*, 173-178.

Sah, V.P., Seasholtz, T.M., Sagi, S.A., and Brown, J.H. (2000). The role of Rho in G protein-coupled receptor signal transduction. Annu Rev Pharmacol Toxicol *40*, 459-489.

Saito, A., Okazaki, H., Sugawara, I., Yamamoto, K., and Takizawa, H. (2003). Potential action of IL-4 and IL-13 as fibrogenic factors on lung fibroblasts in vitro. Int Arch Allergy Immunol *132*, 168-176.

Saito, Y., Gomez, N., Campbell, D.G., Ashworth, A., Marshall, C.J., and Cohen, P. (1994). The threonine residues in MAP kinase kinase 1 phosphorylated by MAP kinase in vitro are also phosphorylated in nerve growth factor-stimulated rat phaeochromocytoma (PC12) cells. FEBS Lett *341*, 119-124.

Sarafi, M.N., Garcia-Zepeda, E.A., MacLean, J.A., Charo, I.F., and Luster, A.D. (1997). Murine monocyte chemoattractant protein (MCP)-5: a novel CC chemokine that is a structural and functional homologue of human MCP-1. J Exp Med *185*, 99-109.

Scarborough, R.M., Naughton, M.A., Teng, W., Hung, D.T., Rose, J., Vu, T.K., Wheaton, V.I., Turck, C.W., and Coughlin, S.R. (1992). Tethered ligand agonist peptides. Structural requirements for thrombin receptor activation reveal mechanism of proteolytic unmasking of agonist function. J Biol Chem *267*, 13146-13149.

Schauwienold, D., Plum, C., Helbing, T., Voigt, P., Bobbert, T., Hoffmann, D., Paul, M., and Reusch, H.P. (2003). ERK1/2-dependent contractile protein expression in vascular smooth muscle cells. Hypertension *41*, 546-552.

Schindler, J.F., Monahan, J.B., and Smith, W.G. (2007). p38 pathway kinases as anti-inflammatory drug targets. J Dent Res *86*, 800-811.

Schini-Kerth, V.B., Bassus, S., Fisslthaler, B., Kirchmaier, C.M., and Busse, R. (1997). Aggregating human platelets stimulate the expression of thrombin receptors in cultured

vascular smooth muscle cells via the release of transforming growth factor-beta1 and platelet-derived growth factorAB. Circulation *96*, 3888-3896.

Schmidt, M., Sun, G., Stacey, M.A., Mori, L., and Mattoli, S. (2003). Identification of circulating fibrocytes as precursors of bronchial myofibroblasts in asthma. J Immunol *171*, 380-389.

Seasholtz, T.M., Majumdar, M., Kaplan, D.D., and Brown, J.H. (1999). Rho and Rho kinase mediate thrombin-stimulated vascular smooth muscle cell DNA synthesis and migration. Circ Res *84*, 1186-1193.

Selman, M., King, T., and Pardo, A. (2001). Idiopathic pulmonary fibrosis: prevailing and evolving hypotheses about its pathogenesis and implications for therapy. Ann Intern Med *134*, 136-151.

Selman, M., Ruiz, V., Cabrera, S., Segura, L., Ramirez, R., Barrios, R., and Pardo, A. (2000). TIMP-1, -2, -3, and -4 in idiopathic pulmonary fibrosis. A prevailing nondegradative lung microenvironment? Am J Physiol Lung Cell Mol Physiol *279*, L562-574.

Serini, G., and Gabbiani, G. (1999). Mechanisms of myofibroblast activity and phenotypic modulation. Exp Cell Res *250*, 273-283.

Seymour, M.L., Zaidi, N.F., Hollenberg, M.D., and MacNaughton, W.K. (2003). PAR1-dependent and independent increases in COX-2 and PGE2 in human colonic myofibroblasts stimulated by thrombin. Am J Physiol Cell Physiol *284*, C1185-1192.

Shankar, R., de la Motte, C.A., Poptic, E.J., and DiCorleto, P.E. (1994). Thrombin receptor-activating peptides differentially stimulate platelet-derived growth factor production, monocytic cell adhesion, and E-selectin expression in human umbilical vein endothelial cells. J Biol Chem *269*, 13936-13941.

Shapiro, M.J., Weiss, E.J., Faruqi, T.R., and Coughlin, S.R. (2000). Protease-activated receptors 1 and 4 are shut off with distinct kinetics after activation by thrombin. J Biol Chem *275*, 25216-25221.

Shi, X., Gangadharan, B., Brass, L.F., Ruf, W., and Mueller, B.M. (2004). Protease-activated receptors (PAR1 and PAR2) contribute to tumor cell motility and metastasis. Mol Cancer Res *2*, 395-402.

Shimizu, S., Gabazza, E.C., Hayashi, T., Ido, M., Adachi, Y., and Suzuki, K. (2000). Thrombin stimulates the expression of PDGF in lung epithelial cells. Am J Physiol Lung Cell Mol Physiol *279*, L503-510.

Shimizu, Y., Dobashi, K., Iizuka, K., Horie, T., Suzuki, K., Tukagoshi, H., Nakazawa, T., Nakazato, Y., and Mori, M. (2001). Contribution of small GTPase Rho and its target protein rock in a murine model of lung fibrosis. Am J Respir Crit Care Med *163*, 210-217.

Shraga-Levine, Z., and Sokolovsky, M. (2000). Functional coupling of G proteins to endothelin receptors is ligand and receptor subtype specific. Cell Mol Neurobiol *20*, 305-317.

Shyy, J.Y., Lin, M.C., Han, J., Lu, Y., Petrime, M., and Chien, S. (1995). The cis-acting phorbol ester "12-O-tetradecanoylphorbol 13-acetate"-responsive element is involved

in shear stress-induced monocyte chemotactic protein 1 gene expression. Proc Natl Acad Sci U S A *92*, 8069-8073.

Shyy, Y.J., Hsieh, H.J., Usami, S., and Chien, S. (1994). Fluid shear stress induces a biphasic response of human monocyte chemotactic protein 1 gene expression in vascular endothelium. Proc Natl Acad Sci U S A *91*, 4678-4682.

Siflinger-Birnboim, A., and Johnson, A. (2003). Protein kinase C modulates pulmonary endothelial permeability: a paradigm for acute lung injury. Am J Physiol Lung Cell Mol Physiol *284*, L435-451.

Sime, P.J., Marr, R.A., Gauldie, D., Xing, Z., Hewlett, B.R., Graham, F.L., and Gauldie, J. (1998). Transfer of tumor necrosis factor-alpha to rat lung induces severe pulmonary inflammation and patchy interstitial fibrogenesis with induction of transforming growth factor-beta1 and myofibroblasts. Am J Pathol *153*, 825-832.

Sime, P.J., Xing, Z., Graham, F.L., Csaky, K.G., and Gauldie, J. (1997). Adenovector-mediated gene transfer of active transforming growth factor-beta1 induces prolonged severe fibrosis in rat lung. J Clin Invest *100*, 768-776.

Simons, K., and Toomre, D. (2000). Lipid rafts and signal transduction. Nat Rev Mol Cell Biol *1*, 31-39.

Singh, I., Knezevic, N., Ahmmed, G.U., Kini, V., Malik, A.B., and Mehta, D. (2007). Galphaq-TRPC6-mediated Ca2+ entry induces RhoA activation and resultant endothelial cell shape change in response to thrombin. J Biol Chem *282*, 7833-7843.

Slepak, V.Z., Katz, A., and Simon, M.I. (1995). Functional analysis of a dominant negative mutant of G alpha i2. J Biol Chem *270*, 4037-4041.

Slepak, V.Z., Quick, M.W., Aragay, A.M., Davidson, N., Lester, H.A., and Simon, M.I. (1993). Random mutagenesis of G protein alpha subunit G(o)alpha. Mutations altering nucleotide binding. J Biol Chem *268*, 21889-21894.

Smith, R.E., Strieter, R.M., Phan, S.H., and Kunkel, S.L. (1996). C-C chemokines: novel mediators of the profibrotic inflammatory response to bleomycin challenge. Am J Respir Cell Mol Biol *15*, 693-702.

Smith, R.E., Strieter, R.M., Phan, S.H., Lukacs, N., and Kunkel, S.L. (1998). TNF and IL-6 mediate MIP-1alpha expression in bleomycin-induced lung injury. J Leukoc Biol *64*, 528-536.

Sokolova, E., Grishina, Z., Buhling, F., Welte, T., and Reiser, G. (2005). Protease-activated receptor-1 in human lung fibroblasts mediates a negative feedback downregulation via prostaglandin E2. Am J Physiol Lung Cell Mol Physiol *288*, L793-802.

Sokolova, E., and Reiser, G. (2007). A novel therapeutic target in various lung diseases: airway proteases and protease-activated receptors. Pharmacol Ther *115*, 70-83.

Soma, Y., Dvonch, V., and Grotendorst, G.R. (1992). Platelet-derived growth factor AA homodimer is the predominant isoform in human platelets and acute human wound fluid. FASEB J *6*, 2996-3001.

Soslau, G., Morgan, D.A., Jaffe, J.S., Brodsky, I., and Wang, Y. (1997). Cytokine mRNA expression in human platelets and a megakaryocytic cell line and cytokine modulation of platelet function. Cytokine *9*, 405-411.

Sower, L.E., Froelich, C.J., Carney, D.H., Fenton, J.W., 2nd, and Klimpel, G.R. (1995). Thrombin induces IL-6 production in fibroblasts and epithelial cells. Evidence for the involvement of the seven-transmembrane domain (STD) receptor for alpha-thrombin. J Immunol *155*, 895-901.

Sridhar, P., Liu, Y., Chin, L.D., Borja, C.E., Mann, M., Skopicki, H.A., and Freter, R.R. (1999). Platelet-derived growth factor-stimulated expression of the MCP-1 immediate-early gene involves an inhibitory multiprotein complex. Mol Cell Biol *19*, 4219-4230.

Srivastava, K.D., and Magazine, H.I. (1998). Thrombin receptor activation inhibits monocyte spreading by induction of ET(B) receptor-coupled nitric oxide release. J Immunol *161*, 5039-5044.

Standiford, T.J., Rolfe, M.R., Kunkel, S.L., Lynch, J.P., 3rd, Becker, F.S., Orringer, M.B., Phan, S., and Strieter, R.M. (1993). Altered production and regulation of monocyte chemoattractant protein-1 from pulmonary fibroblasts isolated from patients with idiopathic pulmonary fibrosis. Chest *103*, 121S.

Steffel, J., Arnet, C., Akhmedov, A., Iseli, S.M., Luscher, T.F., and Tanner, F.C. (2006). Histamine differentially interacts with tumor necrosis factor-alpha and thrombin in endothelial tissue factor induction: the role of c-Jun NH2-terminal kinase. J Thromb Haemost *4*, 2452-2460.

Steinhauser, M.L., Kunkel, S.L., Hogaboam, C.M., Evanoff, H., Strieter, R.M., and Lukacs, N.W. (1998). Macrophage/fibroblast coculture induces macrophage inflammatory protein-1alpha production mediated by intercellular adhesion molecule-1 and oxygen radicals. J Leukoc Biol *64*, 636-641.

Steinhoff, M., Buddenkotte, J., Shpacovitch, V., Rattenholl, A., Moormann, C., Vergnolle, N., Luger, T.A., and Hollenberg, M.D. (2005). Proteinase-activated receptors: transducers of proteinase-mediated signaling in inflammation and immune response. Endocr Rev *26*, 1-43.

Stouffer, G.A., and Runge, M.S. (1998). The role of secondary growth factor production in thrombin-induced proliferation of vascular smooth muscle cells. Semin Thromb Hemost *24*, 145-150.

Strieter, R.M. (2002). Con: Inflammatory mechanisms are not a minor component of the pathogenesis of idiopathic pulmonary fibrosis. Am J Respir Crit Care Med *165*, 1206-1207; discussion 1207-1208.

Strieter, R.M. (2005). Pathogenesis and natural history of usual interstitial pneumonia: the whole story or the last chapter of a long novel. Chest *128*, 526S-532S.

Sugama, Y., Tiruppathi, C., offakidevi, K., Andersen, T.T., Fenton, J.W., 2nd, and Malik, A.B. (1992). Thrombin-induced expression of endothelial P-selectin and intercellular adhesion molecule-1: a mechanism for stabilizing neutrophil adhesion. J Cell Biol *119*, 935-944.

Suganuma, H., Sato, A., Tamura, R., and Chida, K. (1995). Enhanced migration of fibroblasts derived from lungs with fibrotic lesions. Thorax *50*, 984-989.

Sugawara, Y., Nishii, H., Takahashi, T., Yamauchi, J., Mizuno, N., Tago, K., and Itoh, H. (2007). The lipid raft proteins flotillins/reggies interact with Galphaq and are involved in Gq-mediated p38 mitogen-activated protein kinase activation through tyrosine kinase. Cell Signal *19*, 1301-1308.

Sun, H., Charles, C.H., Lau, L.F., and Tonks, N.K. (1993). MKP-1 (3CH134), an immediate early gene product, is a dual specificity phosphatase that dephosphorylates MAP kinase in vivo. Cell *75*, 487-493.

Suo, Z., Wu, M., Ameenuddin, S., Anderson, H.E., Zoloty, J.E., Citron, B.A., Andrade-Gordon, P., and Festoff, B.W. (2002). Participation of protease-activated receptor-1 in thrombin-induced microglial activation. J Neurochem *80*, 655-666.

Suzuki, N., Nakamura, S., Mano, H., and Kozasa, T. (2003). Galpha 12 activates Rho GTPase through tyrosine-phosphorylated leukemia-associated RhoGEF. Proc Natl Acad Sci U S A *100*, 733-738.

Suzuki, T., Moraes, T.J., Vachon, E., Ginzberg, H.H., Huang, T.T., Matthay, M.A., Hollenberg, M.D., Marshall, J., McCulloch, C.A., Abreu, M.T., Chow, C.W., and Downey, G.P. (2005). Proteinase-activated receptor-1 mediates elastase-induced apoptosis of human lung epithelial cells. Am J Respir Cell Mol Biol *33*, 231-247.

Szaba, F.M., and Smiley, S.T. (2002). Roles for thrombin and fibrin(ogen) in cytokine/chemokine production and macrophage adhesion in vivo. Blood *99*, 1053-1059.

Taipale, J., Koli, K., and Keski-Oja, J. (1992). Release of transforming growth factor-beta 1 from the pericellular matrix of cultured fibroblasts and fibrosarcoma cells by plasmin and thrombin. J Biol Chem *267*, 25378-25384.

Tanaka, N., Morita, T., Nezu, A., Tanimura, A., Mizoguchi, I., and Tojyo, Y. (2004). Signaling mechanisms involved in protease-activated receptor-1-mediated interleukin-6 production by human gingival fibroblasts. J Pharmacol Exp Ther *311*, 778-786.

Tang, Y.W., Johnson, J.E., Browning, P.J., Cruz-Gervis, R.A., Davis, A., Graham, B.S., Brigham, K.L., Oates, J.A., Jr., Loyd, J.E., and Stecenko, A.A. (2003). Herpesvirus DNA is consistently detected in lungs of patients with idiopathic pulmonary fibrosis. J Clin Microbiol *41*, 2633-2640.

Tani, K., Ogushi, F., Takahashi, H., Kawano, T., Endo, T., and Sone, S. (1997). Thrombin stimulates platelet-derived growth factor release by alveolar macrophages in rats--significance in bleomycin-induced pulmonary fibrosis. J Med Invest *44*, 59-65.

Tani, K., Yasuoka, S., Ogushi, F., Asada, K., Fujisawa, K., Ozaki, T., Sano, N., and Ogura, T. (1991). Thrombin enhances lung fibroblast proliferation in bleomycin-induced pulmonary fibrosis. Am J Respir Cell Mol Biol *5*, 34-40.

Thannickal, V., and Horowitz, J. (2006). Evolving concepts of apoptosis in idiopathic pulmonary fibrosis. Proc Am Thorac Soc *3*, 350-356.

Thannickal, V.J., Toews, G.B., White, E.S., Lynch, J.P., 3rd, and Martinez, F.J. (2004). Mechanisms of pulmonary fibrosis. Annu Rev Med *55*, 395-417.

Tian, Y., New, D.C., Yung, L.Y., Allen, R.A., Slocombe, P.M., Twomey, B.M., Lee, M.M., and Wong, Y.H. (2004). Differential chemokine activation of CC chemokine receptor 1-

regulated pathways: ligand selective activation of Galpha 14-coupled pathways. Eur J Immunol *34*, 785-795.

Tourkina, E., Hoffman, S., Fenton, J.W., 2nd, Lipsitz, S., Silver, R.M., and Ludwicka-Bradley, A. (2001). Depletion of protein kinase Cepsilon in normal and scleroderma lung fibroblasts has opposite effects on tenascin expression. Arthritis Rheum *44*, 1370-1381.

Treisman, R. (1996). Regulation of transcription by MAP kinase cascades. Curr Opin Cell Biol *8*, 205-215.

Trejo, J., Connolly, A.J., and Coughlin, S.R. (1996). The cloned thrombin receptor is necessary and sufficient for activation of mitogen-activated protein kinase and mitogenesis in mouse lung fibroblasts. Loss of responses in fibroblasts from receptor knockout mice. J Biol Chem *271*, 21536-21541.

Trejo, J., and Coughlin, S.R. (1999). The cytoplasmic tails of protease-activated receptor-1 and substance P receptor specify sorting to lysosomes versus recycling. J Biol Chem *274*, 2216-2224.

Ueda, A., Ishigatsubo, Y., Okubo, T., and Yoshimura, T. (1997). Transcriptional regulation of the human monocyte chemoattractant protein-1 gene. Cooperation of two NF-kappaB sites and NF-kappaB/Rel subunit specificity. J Biol Chem *272*, 31092-31099.

Ueda, A., Okuda, K., Ohno, S., Shirai, A., Igarashi, T., Matsunaga, K., Fukushima, J., Kawamoto, S., Ishigatsubo, Y., and Okubo, T. (1994). NF-kappa B and Sp1 regulate transcription of the human monocyte chemoattractant protein-1 gene. J Immunol *153*, 2052-2063.

Uhal, B.D., Joshi, I., Hughes, W.F., Ramos, C., Pardo, A., and Selman, M. (1998). Alveolar epithelial cell death adjacent to underlying myofibroblasts in advanced fibrotic human lung. Am J Physiol *275*, L1192-1199.

Uhal, B.D., Joshi, I., True, A.L., Mundle, S., Raza, A., Pardo, A., and Selman, M. (1995). Fibroblasts isolated after fibrotic lung injury induce apoptosis of alveolar epithelial cells in vitro. Am J Physiol *269*, L819-828.

Urban, J.D., Clarke, W.P., von Zastrow, M., Nichols, D.E., Kobilka, B., Weinstein, H., Javitch, J.A., Roth, B.L., Christopoulos, A., Sexton, P.M., Miller, K.J., Spedding, M., and Mailman, R.B. (2007). Functional selectivity and classical concepts of quantitative pharmacology. J Pharmacol Exp Ther *320*, 1-13.

van der Poll, T., Levi, M., Hack, C.E., ten Cate, H., van Deventer, S.J., Eerenberg, A.J., de Groot, E.R., Jansen, J., Gallati, H., Buller, H.R., and et al. (1994). Elimination of interleukin 6 attenuates coagulation activation in experimental endotoxemia in chimpanzees. J Exp Med *179*, 1253-1259.

van der Poll, T., van Deventer, S.J., Buller, H.R., Sturk, A., and ten Cate, J.W. (1991). Comparison of the early dynamics of coagulation activation after injection of endotoxin and tumor necrosis factor in healthy humans. Prog Clin Biol Res *367*, 55-60.

Vanhauwe, J.F., Thomas, T.O., Minshall, R.D., Tiruppathi, C., Li, A., Gilchrist, A., Yoon, E.J., Malik, A.B., and Hamm, H.E. (2002). Thrombin receptors activate G(o) proteins in

endothelial cells to regulate intracellular calcium and cell shape changes. J Biol Chem *277*, 34143-34149.

Vauquelin, G., and Van Liefde, I. (2005). G protein-coupled receptors: a count of 1001 conformations. Fundam Clin Pharmacol *19*, 45-56.

Vergnolle, N., Cellars, L., Mencarelli, A., Rizzo, G., Swaminathan, S., Beck, P., Steinhoff, M., Andrade-Gordon, P., Bunnett, N.W., Hollenberg, M.D., Wallace, J.L., Cirino, G., and Fiorucci, S. (2004). A role for proteinase-activated receptor-1 in inflammatory bowel diseases. J Clin Invest *114*, 1444-1456.

Vogel, S.M., Gao, X., Mehta, D., Ye, R.D., John, T.A., Andrade-Gordon, P., Tiruppathi, C., and Malik, A.B. (2000). Abrogation of thrombin-induced increase in pulmonary microvascular permeability in PAR-1 knockout mice. Physiol Genomics *4*, 137-145.

Vogt, S., Grosse, R., Schultz, G., and Offermanns, S. (2003). Receptor-dependent RhoA activation in G12/G13-deficient cells: genetic evidence for an involvement of Gq/G11. J Biol Chem *278*, 28743-28749.

Vu, T.K., Hung, D.T., Wheaton, V.I., and Coughlin, S.R. (1991). Molecular cloning of a functional thrombin receptor reveals a novel proteolytic mechanism of receptor activation. Cell *64*, 1057-1068.

Walz, D.A., Anderson, G.F., Ciaglowski, R.E., Aiken, M., and Fenton, J.W., 2nd. (1985). Thrombin-elicited contractile responses of aortic smooth muscle. Proc Soc Exp Biol Med *180*, 518-526.

Wang, H., Ubl, J.J., Stricker, R., and Reiser, G. (2002). Thrombin (PAR-1)-induced proliferation in astrocytes via MAPK involves multiple signaling pathways. Am J Physiol Cell Physiol *283*, C1351-1364.

Wang, J., Gigliotti, F., Bhagwat, S.P., Maggirwar, S.B., and Wright, T.W. (2007). Pneumocystis stimulates MCP-1 production by alveolar epithelial cells through a JNK-dependent mechanism. Am J Physiol Lung Cell Mol Physiol *292*, L1495-1505.

Wang, L., Luo, J., Fu, Y., and He, S. (2006). Induction of interleukin-8 secretion and activation of ERK1/2, p38 MAPK signaling pathways by thrombin in dermal fibroblasts. Int J Biochem Cell Biol *38*, 1571-1583.

Wang, R., Ramos, C., Joshi, I., Zagariya, A., Pardo, A., Selman, M., and Uhal, B.D. (1999). Human lung myofibroblast-derived inducers of alveolar epithelial apoptosis identified as angiotensin peptides. Am J Physiol *277*, L1158-1164.

Ware, L.B., Matthay, M.A., Parsons, P.E., Thompson, B.T., Januzzi, J.L., and Eisner, M.D. (2007). Pathogenetic and prognostic significance of altered coagulation and fibrinolysis in acute lung injury/acute respiratory distress syndrome. Crit Care Med *35*, 1821-1828.

Wennerberg, K., and Der, C.J. (2004). Rho-family GTPases: it's not only Rac and Rho (and I like it). J Cell Sci *117*, 1301-1312.

Wennerberg, K., Rossman, K.L., and Der, C.J. (2005). The Ras superfamily at a glance. J Cell Sci *118*, 843-846.

Wess, J. (1998). Molecular basis of receptor/G-protein-coupling selectivity. Pharmacol Ther *80*, 231-264.

Wettschureck, N., and Offermanns, S. (2005). Mammalian G proteins and their cell type specific functions. Physiol Rev *85*, 1159-1204.

Whyte, M., Hubbard, R., Meliconi, R., Whidborne, M., Eaton, V., Bingle, C., Timms, J., Duff, G., Facchini, A., Pacilli, A., Fabbri, M., Hall, I., Britton, J., Johnston, I., and Di Giovine, F. (2000). Increased risk of fibrosing alveolitis associated with interleukin-1 receptor antagonist and tumor necrosis factor-alpha gene polymorphisms. Am J Respir Crit Care Med *162*, 755-758.

Widmann, C., Gibson, S., Jarpe, M.B., and Johnson, G.L. (1999). Mitogen-activated protein kinase: conservation of a three-kinase module from yeast to human. Physiol Rev *79*, 143-180.

Wilborn, J., Crofford, L.J., Burdick, M.D., Kunkel, S.L., Strieter, R.M., and Peters-Golden, M. (1995). Cultured lung fibroblasts isolated from patients with idiopathic pulmonary fibrosis have a diminished capacity to synthesize prostaglandin E2 and to express cyclooxygenase-2. J Clin Invest *95*, 1861-1868.

Wilkinson, S.E., Parker, P.J., and Nixon, J.S. (1993). Isoenzyme specificity of bisindolylmaleimides, selective inhibitors of protein kinase C. Biochem J *294 (Pt 2)*, 335-337.

Willis, B.C., and Borok, Z. (2007). TGF-beta-induced EMT: mechanisms and implications for fibrotic lung disease. Am J Physiol Lung Cell Mol Physiol *293*, L525-534.

Willis, B.C., Liebler, J.M., Luby-Phelps, K., Nicholson, A.G., Crandall, E.D., du Bois, R.M., and Borok, Z. (2005). Induction of epithelial-mesenchymal transition in alveolar epithelial cells by transforming growth factor-beta1: potential role in idiopathic pulmonary fibrosis. Am J Pathol *166*, 1321-1332.

Wu, S.Q., and Aird, W.C. (2005). Thrombin, TNF-alpha, and LPS exert overlapping but nonidentical effects on gene expression in endothelial cells and vascular smooth muscle cells. Am J Physiol Heart Circ Physiol *289*, H873-885.

Xing, L., and Remick, D.G. (2007). Promoter elements responsible for antioxidant regulation of MCP-1 gene expression. Antioxid Redox Signal *9*, 1979-1989.

Yan, W., Tiruppathi, C., Lum, H., Qiao, R., and Malik, A.B. (1998). Protein kinase C beta regulates heterologous desensitization of thrombin receptor (PAR-1) in endothelial cells. Am J Physiol *274*, C387-395.

Yang, J., Wu, J., Jiang, H., Mortensen, R., Austin, S., Manning, D.R., Woulfe, D., and Brass, L.F. (2002). Signaling through Gi family members in platelets. Redundancy and specificity in the regulation of adenylyl cyclase and other effectors. J Biol Chem *277*, 46035-46042.

Yang, Y.H., Hall, P., Little, C.B., Fosang, A.J., Milenkovski, G., Santos, L., Xue, J., Tipping, P., and Morand, E.F. (2005). Reduction of arthritis severity in protease-activated receptor-deficient mice. Arthritis Rheum *52*, 1325-1332.

Yasui, H., Gabazza, E.C., Taguchi, O., Risteli, J., Risteli, L., Wada, H., Yuda, H., Kobayashi, T., Kobayashi, H., Suzuki, K., and Adachi, Y. (2000). Decreased protein C activation is associated with abnormal collagen turnover in the intraalveolar space of patients with interstitial lung disease. Clin Appl Thromb Hemost *6*, 202-205.

Yasui, H., Gabazza, E.C., Tamaki, S., Kobayashi, T., Hataji, O., Yuda, H., Shimizu, S., Suzuki, K., Adachi, Y., and Taguchi, O. (2001). Intratracheal administration of activated protein C inhibits bleomycin-induced lung fibrosis in the mouse. Am J Respir Crit Care Med *163*, 1660-1668.

Yoshida, K., Kuwano, K., Hagimoto, N., Watanabe, K., Matsuba, T., Fujita, M., Inoshima, I., and Hara, N. (2002). MAP kinase activation and apoptosis in lung tissues from patients with idiopathic pulmonary fibrosis. J Pathol *198*, 388-396.

Zavadil, J., and Bottinger, E.P. (2005). TGF-beta and epithelial-to-mesenchymal transitions. Oncogene *24*, 5764-5774.

Zhang, H.Y., Gharaee-Kermani, M., Zhang, K., Karmiol, S., and Phan, S.H. (1996). Lung fibroblast alpha-smooth muscle actin expression and contractile phenotype in bleomycin-induced pulmonary fibrosis. Am J Pathol *148*, 527-537.

Zhang, K., Gharaee-Kermani, M., Jones, M.L., Warren, J.S., and Phan, S.H. (1994). Lung monocyte chemoattractant protein-1 gene expression in bleomycin-induced pulmonary fibrosis. J Immunol *153*, 4733-4741.

Zhang, L., Keane, M.P., Zhu, L.X., Sharma, S., Rozengurt, E., Strieter, R.M., Dubinett, S.M., and Huang, M. (2004). Interleukin-7 and transforming growth factor-beta play counter-regulatory roles in protein kinase C-delta-dependent control of fibroblast collagen synthesis in pulmonary fibrosis. J Biol Chem *279*, 28315-28319.

Zhang, X., and Godbey, W.T. (2006). Viral vectors for gene delivery in tissue engineering. Adv Drug Deliv Rev *58*, 515-534.

Zhang, Y., Lee, T.C., Guillemin, B., Yu, M.C., and Rom, W.N. (1993). Enhanced IL-1 beta and tumor necrosis factor-alpha release and messenger RNA expression in macrophages from idiopathic pulmonary fibrosis or after asbestos exposure. J Immunol *150*, 4188-4196.

Zhou, X., Hu, H., Huynh, M.L., Kotaru, C., Balzar, S., Trudeau, J.B., and Wenzel, S.E. (2007). Mechanisms of tissue inhibitor of metalloproteinase 1 augmentation by IL-13 on TGF-beta 1-stimulated primary human fibroblasts. J Allergy Clin Immunol *119*, 1388-1397.

Zorzetto, M., Ferrarotti, I., Trisolini, R., Agli, L.L., Scabini, R., Novo, M., De Silvestri, A., Patelli, M., Martinetti, M., Cuccia, M., Poletti, V., Pozzi, E., and Luisetti, M. (2003). Complement receptor 1 gene polymorphisms are associated with idiopathic pulmonary fibrosis. Am J Respir Crit Care Med *168*, 330-334.

Zuo, F., Kaminski, N., Eugui, E., Allard, J., Yakhini, Z., Ben-Dor, A., Lollini, L., Morris, D., Kim, Y., DeLustro, B., Sheppard, D., Pardo, A., Selman, M., and Heller, R.A. (2002). Gene expression analysis reveals matrilysin as a key regulator of pulmonary fibrosis in mice and humans. Proc Natl Acad Sci U S A *99*, 6292-6297.

APPENDIX

HABP	C-terminal sequence	IC_{50} [nM]
Gq	L Q L N L K E Y N L V	320
T2-14	L Q L N L K K Y N R V	106
B2AR-x	Q R L H L R G Y E F L	8277
RHO-8	L L E N L R D C G M F	8411

Table I. IC_{50} Values for high affinity C-terminal PAR_1 receptor high affinity binding proteins (HABP). Using competitive ELISA the affinity of selected purified MBP-C-terminal fusion proteins for activated PAR_1 was determined. β2AR-x contains a sequence obtained from screening the activated β2-adrenergic receptor with a Gαs library, and there is either no binding or very low-affinity binding. There is also little or no binding with RHO8, a peptide identified from a Gαt library for its ability to bind activated Rhodopsin. This support the selective nature of the binding of peptide sequences to PAR_1.

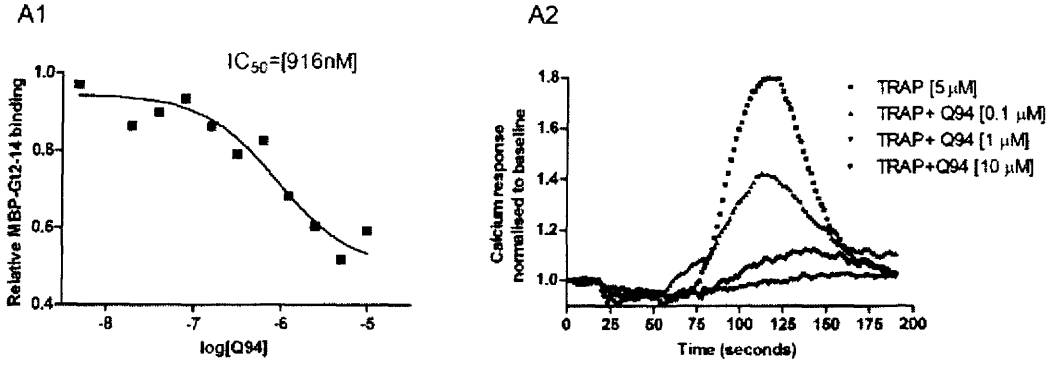


Figure A. Q94 displaces the high affinity binding peptide MBP-T2-14 from activated PAR_1 and blocks thrombin induced Ca^{2+} transients in HEK293 cells.

For the binding studies (left panel, A1), SF9 cells were treated with [100 nM] TRAP. Purified T2-14 fused with *E. coli* maltose binding protein (MBP-T2-14) [100 nM] was added to wells and allowed to bind to the receptor. Increasing amounts of Q94 were added to wells in triplicate, binding of MBP-T2-14 was measured using an ELISA for MBP. Data presented are the mean of individual wells from 3 separate experiments. A non-linear regression analysis was generated using GraphPad Prism, version 5.0. For the Ca^{2+} transient studies (right panel, A2), assays were performed using Molecular Devices Calcium Plus. TRAP: thrombin receptor activating peptide; MBP: maltose binding protein.

Publications arising from this thesis:

Full papers:

1. Thrombin induces fibroblast CCL2/JE production and release via coupling of PAR_1 to $G\alpha_q$ and cooperation between ERK1/2 and Rho kinase signalling pathways. ***X Deng,*** *PF. Mercer, CJ. Scotton, A Gilchrist and RC Chambers:* ***Molecular Biology of the Cell*** *2008,* 6, 2520-2533.

2. Signaling pathways involved in proteinase-activated receptor1-induced proinflammatory and profibrotic mediator release following lung injury. *PF. Mercer,* ***Deng X*** *and RC. Chambers.* ***Ann N Y Acad Sci****. 2007, 1096:86-8.*

3. The injured epithelium is a prominent source of PAR_1 inducible CCL2 in pulmonary fibrosis. *PF Mercer, Johns RH, Scotton CJ, Krupiczojc MA, Koenigshoff M,* ***Deng X,*** *Howell D.CJ, McAnulty RJ, Das A, Eickelberg O and Chambers RC. (manuscript in revision with* ***American Journal of Respiratory and Critical Care Medicine)***

Abstracts:

1. "Thrombin induces fibroblast CCL2/JE production and release via coupling of PAR_1 to $G\alpha_q$ and cooperation between ERK1/2 and Rho kinase signalling pathways". ***Deng XL,*** *P.F. Mercer, Chris J Scotton, A. Cilchrist and R.C. Chambers.* ***6th ERS Lung Science Conference, 2008.***

2. "Identification of PAR_1-G protein signaling pathways involved in thrombin-induced CCL2 release". ***Deng XL,*** *P.F. Mercer, Chris J Scotton, G.J. Laurent and R.C. Chambers.* ***America Thoracic Society (ATS) conference, 2007****.*

3. "Identification of PAR_1-G protein signaling pathways involved in thrombin-induced CCL2 release". ***Deng XL,*** *P.F. Mercer, Chris J Scotton, G.J. Laurent and R.C. Chambers.* ***14th international colloquium on lung fibrosis (ICLF), Germany, September 2006***

4. "Identification of PAR_1-G protein signaling pathways involved in thrombin-induced CCL2 release" Poster discussion session. ***Deng XL,*** *P.F. Mercer, G.J. Laurent and R.C. Chambers.* ***Cell Signaling World 2006, Luxemburg, January 2006***

5. "Identification of PAR_1-G protein signaling pathways involved in thrombin induced

CCL2 release". ***Deng XL**, P.F. Mercer, G.J. Laurent and R.C. Chambers.* ***Thorax, 60, Supl.2, ii789, December, 2005***

Academic Awards

1. **Bursary awarded for the 6th European Respiratory Society (ERS) lung science conference.** *Portugal, March 2008.*

2. **Dorothy Hodgkin Postgraduate Award (DHPA) Sep.2004-Sep.2007: PhD Studentship, University College London.** *Founded by Medical Research Council in United Kingdom and Hutchison Whampoa Company.*

3. **Travel funding awarded by Roberts Skills Training Programme, UCL. 2006 & 2007**

4. **1st poster prize, 14th international colloquium on lung fibrosis (ICLF)** *Germany, September 2006.*

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Thrombin Induces Fibroblast CCL2/JE Production and Release via Coupling of PAR_1 to $G\alpha_q$ and Cooperation between ERK1/2 and Rho Kinase Signaling Pathways

Xiaoling Deng,* Paul F. Mercer,* Chris J. Scotton,* Annette Gilchrist,† and Rachel C. Chambers*

*Centre for Respiratory Research, University College London, London WC1E 6JJ, United Kingdom; and †Caden Biosciences, Madison, WI 53711

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Uncontrolled activation of the coagulation cascade after tissue injury has been implicated in both inflammation and tissue fibrosis. Thrombin exerts pluripotent cellular effects via its high-affinity receptor, proteinase-activated receptor-1 (PAR_1) and signaling via $G\alpha_{i/o}$, $G\alpha_q$, or $G\alpha_{12/13}$. Activation of PAR_1 on fibroblasts, a key effector cell in fibrosis, results in the induction of several mediators, including the potent monocyte and fibrocyte chemoattractant CCL2. The aim of this study was to identify the G protein and signaling pathway involved in PAR_1-mediated CCL2 production and release. Using a novel PAR_1 antagonist that blocks the interaction between PAR_1 and $G\alpha_q$, we report for the first time that PAR_1 coupling to $G\alpha_q$ is essential for thrombin-induced CCL2 gene expression and protein release in murine lung fibroblasts. We further demonstrate that these effects are mediated via the cooperation between ERK1/2 and Rho kinase signaling pathways: a calcium-independent protein kinase C (PKC), c-Raf, and ERK1/2 pathway was found to mediate PAR_1-induced CCL2 gene transcription, whereas a phospholipase C, calcium-dependent PKC, and Rho kinase pathway influences CCL2 protein release. We propose that targeting the interaction between PAR_1 and $G\alpha_q$ may allow us to selectively interfere with PAR_1 proinflammatory and profibrotic signaling, while preserving the essential role of other PAR_1-mediated cellular responses.

INTRODUCTION

Inflammation and the subsequent fibroproliferative response are critical components of tissue repair after injury. However, if uncontrolled, these processes can lead to the development of tissue remodeling and fibrosis of the skin, vasculature, and internal organs, including the lung. Previously, the fibroblast was considered to be a passive participant in tissue repair through its end-stage contribution of extracellular matrix synthesis. However, emerging evidence now points to a more active role for fibroblasts in the response to tissue injury by releasing a host of mediators, including the CC-chemokine: CCL2 (MCP-1/CCL2/JE; Hogaboam *et al.*, 1998). Although primarily considered a potent chemoattractant for monocytes, T-cells, and natural killer cells, CCL2 is also involved in the direct activation of fibroblasts leading to extracellular matrix generation via the induction of the potent profibrotic mediator, transforming growth factor β1 (TGF-β1; Gharaee-Kermani *et al.*, 1996). Recent evidence further suggests that CCL2 may also contribute to excessive collagen deposition via the recruitment of fibrocytes (Moore *et al.*, 2005), which are believed to represent a source of fibroblasts and myofibroblasts during the fibroproliferative response to tissue damage (Phillips *et al.*, 2004).

One of the earliest responses to tissue injury involves the highly coordinated activation of the coagulation cascade with the resultant generation of thrombin. In addition to its central role in hemostasis, thrombin exerts a number of cellular effects that initiate and influence subsequent inflammatory and tissue repair responses (Chambers, 2003). Thrombin exerts potent profibrotic effects by influencing fibroblast function and has also been shown to up-regulate CCL2 expression by several cell types, including monocytes (Colotta *et al.*, 1994), endothelial cells (Colotta *et al.*, 1994; Marin *et al.*, 2001), smooth muscle cells (Brandes *et al.*, 2001), and dermal fibroblasts (Bachli *et al.*, 2003). The cellular effects of thrombin are largely, but not exclusively, mediated via the activation of a unique family of cell surface receptors termed, proteinase-activated receptors (PARs). To date, four PARs have been described, of which three (PAR_1, PAR_3, and PAR_4) are activated by thrombin. These receptors belong to the seven transmembrane domain G protein–coupled receptor (GPCR) superfamily, but are activated by a unique mechanism involving limited proteolytic cleavage of the N-terminal extracellular domain leading to the unmasking of a tethered ligand that in turn activates the receptor by intramolecular binding (Vu *et al.*, 1991) .

PAR_1 is the high-affinity thrombin receptor and the major receptor responsible for mediating many of the proinflammatory and profibrotic effects of thrombin via the induction and activation of a host of secondary mediators(Chambers, 2003). PAR_1 exhibits the ability to couple to multiple G

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Address correspondence to: Rachel C. Chambers (r.chambers@ucl.ac.uk).

Abbreviations used: ARDS, acute respiratory distress syndrome; BALF, bronchoalveolar lavage fluid; ca-MEK1, constitutively active MEK1; dn-MEK1, dominant negative-MEK1; EGFP, enhanced green fluorescent protein; ILD, interstitial lung disease; IPF, idiopathic pulmonary fibrosis; KO, knockout; MLF, mouse lung fibroblast; PAR, proteinase-activated receptor; wt-MEK1, wild-type MEK1.

protein family subunits, including $G\alpha_{i/o}$, $G\alpha_q$, or $G\alpha_{12/13}$ within the same cell type. In general, the $G\alpha_{i/o}$ pathway inhibits adenylate cyclase and the generation of cyclic adenosine monophosphate (cAMP); the $G\alpha_q$ pathway involves phospholipase C-β (PLC-β) activation and concomitant calcium mobilization and protein kinase C (PKC) activation, whereas the $G\alpha_{12/13}$ pathway activates Rho kinase and regulates actin remodeling (Coughlin, 2000).

Studies employing PAR_1 antagonists and PAR_1-deficient mice have provided strong evidence that PAR_1 signaling plays an important role in inflammation and tissue remodeling in a number of tissues, including the vasculature (Cheung *et al.*, 1999), the kidney (Cunningham *et al.*, 2000), the liver (Fiorucci *et al.*, 2004), and the lung (Howell *et al.*, 2005). In the context of lung injury, we have recently reported that protection from bleomycin-induced lung inflammation and fibrosis in PAR_1-deficient mice is accompanied by a marked attenuation of the characteristic increase in CCL2 lung levels (Howell *et al.*, 2005). In human fibroproliferative lung diseases, CCL2 levels in bronchoalveolar lavage fluid (BALF) correlate with the severity of lung injury in acute respiratory distress syndrome (ARDS; Goodman *et al.*, 1996). CCL2 lung levels are also increased in patients with interstitial lung disease (ILD) and in patients with the fatal chronic fibrotic lung condition, idiopathic pulmonary fibrosis (IPF), where CCL2 may serve as a useful biomarker for the clinical course of the disease (Suga *et al.*, 1999). In patients with fibrotic lung disease and in animal models, fibroblast numbers are dramatically increased, and numerous cells are strongly immunoreactive for both CCL2 and PAR_1 (Mercer, Johns, Scotton, Krupiczojc, Koenigshoff, Howell, McAnulty, Das, Eickelberg, and Chambers, unpublished data). However, the signaling pathways involved in PAR_1-mediated CCL2 production remain poorly understood. The aim of this study was therefore to begin to delineate the signaling pathways by which thrombin induces CCL2 production by lung fibroblasts. We report for the first time that thrombin induces fibroblast CCL2 production and release via coupling of PAR_1 to $G\alpha_q$ and the cooperation between ERK1/2 and Rho kinase signaling pathways. We further provide evidence that the Ca^{2+}-independent PKC, c-Raf, and ERK1/2 pathway is responsible for thrombin-induced CCL2 gene expression, whereas the second PLC, Ca^{2+}, Ca^{2+}-dependent PKC, Rho kinase pathway influences CCL2 protein release via a posttranscriptional mechanism. These data demonstrate a central role for $G\alpha_q$ in thrombin-induced CCL2 signaling. In this report, we further demonstrate the effectiveness of blocking this response using a recently developed novel small-molecule PAR_1 antagonist, which blocks PAR_1 at the intracellular interaction site with $G\alpha_q$ (Caden Biosciences, Madison, WI). This antagonist may allow more selective targeting of some, but not all, PAR_1-mediated cellular responses and may hold promise for interfering with deleterious PAR_1 signaling in a number of inflammatory and fibrotic conditions associated with uncontrolled activation of the coagulation cascade.

MATERIALS AND METHODS

Materials

Human thrombin, Ro-318425, GF109203X, Gö6976, U0126, SB 203580, Y-27632, H-1152, U73122, BAPTA-AM, and c-Raf inhibitor were purchased from Calbiochem (Merck Biosciences, Nottingham, United Kingdom). Tumor necrosis factor α (TNF-α) was purchased from Peprotech (London, United Kingdom). Anti-phospho-p38, anti-p38, anti-phospho-ERK1/2, anti-ERK1/2, anti-phospho-c-Raf, anti-c-Raf, and anti-MLC antibodies were purchased from New England Biolabs (Hitchin, United Kingdom). Anti-phospho-MLC antibody was a kind and generous gift from Dr. James M. Staddon (Eisai London Research Laboratories, United Kingdom). Anti-CCL2 antibody was obtained from R&D Systems (Abingdon, United Kingdom). Wild-type MEK1 (wt-MEK1), dominant negative MEK1 (dn-MEK1), and constitutively active MEK1 (ca-MEK1) cloned to pBABEpuro eukaryotic expression vectors were generous gifts from Professor Chris Marshall (Cancer Research UK, London, United Kingdom). Selective PAR_1 agonists, corresponding to the sequence TFLLR-NH_2 (TF) and the reverse control peptide RLLFT-NH_2 (RL), were obtained from Dr. Robert P. Mecham (University of Washington Medical School, St. Louis, MO). pRev Tet-on vector was purchased from BD Biosciences (San Jose, CA). The pRevTRE2-dEGFP (a control vector encoding enhanced green fluorescent protein [EGFP]), pRevTRE2-$G\alpha_q$, pRevTRE2-$G\alpha_{12}$, pRevTRE-$G\alpha_{13}$, and pRevTRE-$G\alpha_{i/o}$ minigenes and the PAR_1 antagonist Q94 were developed by Dr. Annette Gilchrist (Caden Biosciences). These C-terminal G alpha minigenes encode 11 amino acid C-terminal sequences of $G\alpha_q$, $G\alpha_{12}$, $G\alpha_{13}$, and $G\alpha_i$, which act as highly specific competitive inhibitors for each isoform (Gilchrist *et al.*, 2001, 2002). The novel PAR_1 antagonist, Q94, is a small molecule (MW <500) that meets the Lipinski rule of five and was identified during an ELISA screen for competition of a high-affinity peptide that mimics the C-terminus of $G\alpha_q$ using a commercially available library (ChemDiv). Further details of the Q94 selection criteria and binding affinity for PAR_1 are provided as supplementary data (see Supplementary Data Table S1 and Figure 1). A patent application for this compound has been filed (patent application EFS ID 2841714; application number 61027665) This compound will be made freely available to qualified investigators upon application to Dr. A. Gilchrist (Caden Biosciences).

Fibroblast Culture

Mouse lung fibroblasts (MLFs) from PAR_1 knockout (PAR_1 KO) and corresponding wild-type MLFs were a kind gift from Professor Shaun Coughlin (University of California, San Francisco, CA) and have been described previously (Trejo *et al.*, 1996). Cells were maintained in DMEM supplemented with penicillin (100 U/ml), glutamine (100 U/ml), streptomycin (100 U/ml), and 10% (vol/vol) FCS (DMEM, 10% FCS), in a humidified atmosphere containing 10% CO_2. Cells were routinely passaged every 5–6 d. There were no noticeable effects on the parameters measured for cells used between passages 8 and 20. Cells were routinely tested and found negative for mycoplasma infection. For all experiments, cells were grown to confluence and 0.01% serum-starved for 24 h before stimulation.

Detection of CCL2 by ELISA

Cells were seeded in 96-well plates (Nunc, Naperville, IL). For each condition there were three biological replicates, and after the specified period, supernatants from each replicate were evaluated for CCL2 levels in duplicate by sandwich ELISA according to the manufacturer's instructions (BD Biosciences, Bath, United Kingdom). The lowest detection limit of the assay was 15.6 pg ml^{-1}, and the standard curve was linear up to 1000 pg ml^{-1}.

Western Blotting of Phosphorylated Kinases and Proteins

MLFs monolayers from six-well plates were washed twice with ice-cold phosphate-buffered saline (PBS), and cells were lysed by adding 100 μl Laemmli sample buffer directly to the monolayer, followed by scraping with a cell scraper. The cell lysate was passed through a 2-gauge needle several times to shear DNA and heated for 10 min at 85°C. Proteins from each lysate were separated by electrophoresis on a 10% or 12.5% SDS-polyacrylamide gel with a 7% stacking gel. Separated proteins were transferred onto Hybond-ECL nylon membranes (GE Healthcare, Waukesha, WI). The membranes were incubated with various primary antibodies: anti-phospho-p38 (1/1000), anti-p38 (1/2500), anti-phospho-ERK (1/2000), anti-ERK (1/2500), anti-phospho-Raf (1/2500), anti-Raf (1/2500), anti-phospho-MLC (1/500), and anti-MLC (1/1000) overnight at 4°C. A horseradish peroxidase–conjugated anti-rabbit IgG (DAKO, Cambridge, United Kingdom) was added at a 1:5000 dilution for 1 h at room temperature. Immunoreactive bands were visualized by standard enhanced chemiluminescence detection (Amersham Pharmacia Biotech, Piscataway, NJ).

Quantitative Real-Time RT-PCR Analysis of CCL2 mRNA Levels

Total RNA from cell cultures was isolated with TRIzol reagent per the manufacturer's protocol. RNA was DNase-treated using a DNAfree kit (Ambion, Europe, Ltd, Huntingdon, United Kingdom). Random hexamers were used as the primer for reverse transcription (RT) of 1 μg of total RNA in a reaction volume of 20 μl using the Applied Biosystems Geneamp RNA PCR core kit (Applied Biosystems, United Kingdom) following the manufacturer's instructions. Real-time RT-PCR was conducted using the Platinum SYBR Green qPCR SuperMix UDG (Invitrogen, Paisley, United Kingdom) on a LightCycler 1.5 Real-Time Detection System (Roche, Lewes, United Kingdom) and analyzed using LightCycler Real-time PCR Detection System (software version 3.5). Cycling conditions were as follows: one cycle of 50°C (2 min) and 95°C (2 min) and 45 cycles of 95°C (5 s), 55°C (5 s), and 72°C (15 s). The specificity of the PCR product was confirmed by melting curve analysis and gel electrophoresis. Relative quantitation was performed using the $2^{-}\Delta\Delta Cp$

method, with 18S as the reference gene. The CCL2 primers were as follows: 5'-AGCTCTCTCTTCCTCCACCAC-3' and 3'-CGTTAACTGCATCTG-GCTGA-5'.

Retroviral Transduction with Mutant MEK1 Constructs

Ecotropic Phoenix packaging cells were transiently transfected with either wild-type MEK1, dominant negative MEK1, or constitutively active MEK1 using a calcium phosphate precipitation transfection protocol. Virus containing supernatants were harvested and used to infect target MLFs as previously described (Janes and Watt, 2004). Stably transduced MLFs were grown in the presence of puromycin (2.5 μg/ml; Calbiochem, Nottingham, United Kingdom).

Retroviral Transduction with G Protein Minigenes

Ecotropic Phoenix packaging cells were transiently transfected with either pRevTet-On vector or pRevTRE2 minigenes (pRevTRE2-EGFP and pRevTRE2-Gα_q) according to the instructions from the manufacturer (CLONTECH, Palo Alto, CA), and virus-containing supernatants were used to infect and transducer target MLFs. Transduced Tet-On-MLFs were selected in medium containing 400 μg/ml G418. Tet-On-MLFs were subsequently infected with pRevTRE2-EGFP, pRevTRE2-Gα_q, pRevTRE2-Gα_{12}, pRevTRE2-Gα_{13}, or pRevTRE2-Gα_i minigenes containing viral supernatants. The Tet-On-MLFs transduced with EGFP or G protein minigenes were selected by culture in medium containing 400 μg/ml G418 and 500 μg/ml hygromycin. Expression of EGFP or minigenes was induced by incubation with 2 μg/ml doxycycline. Subsequent experiments were performed 48 h after doxycycline addition. Transduction efficiency was ~90% as determined by assessing the expression obtained with the EGFP encoding RevTRE2-EGFP vector.

Immunocytofluoresence

MLFs were plated on eight-well chamber glass slides. After stimulation, cells were rinsed with PBS and then fixed for 10 min with 4% formaldehyde in PBS and permeabilized with 0.2% Triton X-100 at room temperature. To avoid nonspecific binding, the cells were incubated with 4% rabbit normal serum for 1 h at room temperature and then washed three times with PBS. Primary antibody (goat polyclonal anti-CCL2; R&D Systems, Europe, Ltd, Abingdon, United Kingdom) was incubated for 1 h at room temperature after three washes with PBS. Secondary antibody (FITC-conjugated rabbit anti-goat IgG) was incubated in the dark for 1 h at room temperature, and subsequently slides were washed three times with PBS. Immunofluoresence images were acquired by confocal microscopy using the Bio-Rad MRC 1024 confocal system (Hercules, CA), and the images were analyzed for the pixel intensity of the fluorescence using the Bio-Rad LaserPix image analysis software.

Statistical Analysis

Data were analyzed by two-tailed Student's *t* test for single and by one-way analysis of variance with the Newman-Keuls post hoc analysis for multiple group comparisons. Differences were considered significant at $p < 0.05$.

RESULTS

Thrombin Induces CCL2 Production and CCL2 mRNA Accumulation in Murine Lung Fibroblasts (MLFs)

To determine the effect of thrombin on MLF CCL2 production and release, MLFs were exposed to various concentrations of thrombin, and CCL2 protein levels in culture supernatants were assessed by ELISA. Figure 1, A and B, shows that thrombin stimulates CCL2 protein release in a time- and dose-dependent manner from 0.03 nM onward. CCL2 production continued to increase at all concentrations examined, and the effect did not plateau at the highest concentration of thrombin (300 nM) examined. Time-course experiments (Figure 1B) with thrombin at a physiologically relevant concentration (10 nM) showed that the sharpest increase in CCL2 release occurs over the first 12 h.

To determine whether thrombin influences CCL2 gene expression, the effect of thrombin on CCL2 mRNA levels

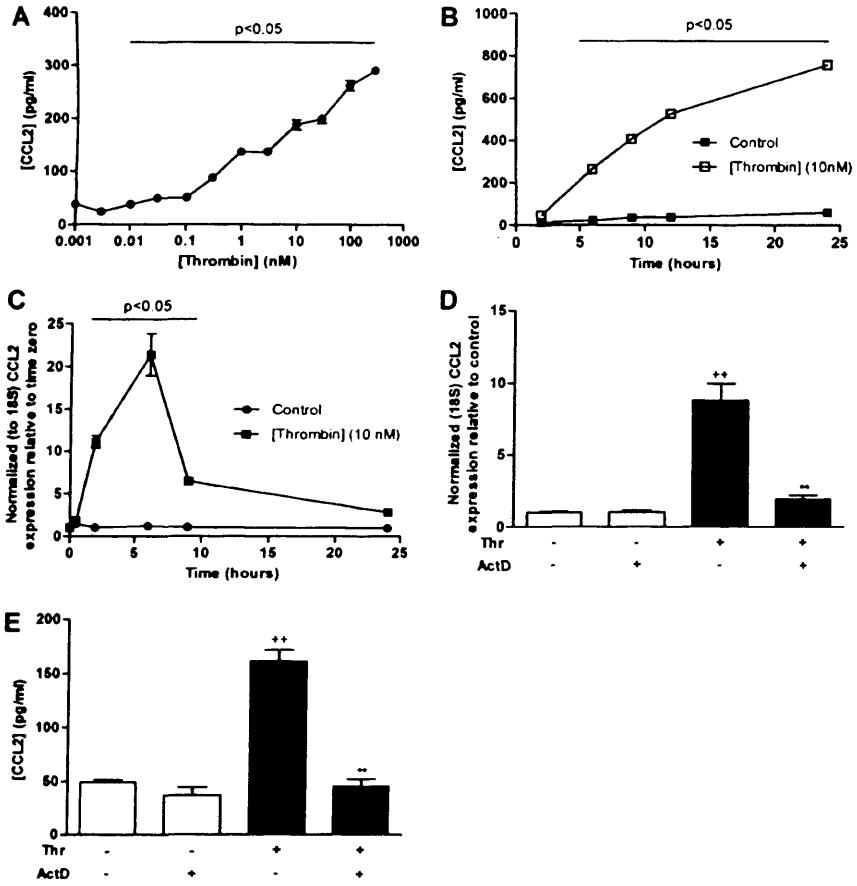

Figure 1. Thrombin stimulates fibroblast CCL2 gene expression and protein production. (A and B) Dose–response (A) and time-course (B) data for the effect of thrombin on MLF CCL2 protein release. MLFs were exposed to thrombin (0.001–300 nM) for 6 h or to 10 nM thrombin for varying durations (2–24 h). Supernatants from cell cultures after incubation were analyzed for CCL2 protein secretion by ELISA. (C) Time-course data for the effect of thrombin on CCL2 mRNA levels. MLFs were exposed to serum-free control medium (DMEM) or thrombin (10 nM) for incubation times from 0.5 to 24 h. CCL2 mRNA levels at each time point were assessed by quantitative real time RT-PCR. Data are expressed as fold change relative to time zero for each time point (mean ± SEM from triplicates) after normalization to 18S RNA. (D) The effect ActD on thrombin-induced CCL2 mRNA levels. MLFs were exposed to thrombin for 2 h with or without preincubation with ActD (1 μg/ml) for 30 min. CCL2 mRNA levels were determined as in C. (E) The effect of ActD on thrombin-induced CCL2 protein release. MLFs were preincubated with ActD (1 μg/ml) for 30 min before exposure to thrombin for 6 h. CCL2 release into cell culture were analyzed by ELISA as in A. Data are expressed as the mean ± SEM from triplicates. $p < 0.05$, comparison with unstimulated cells or time point-matched media control cells; $^{++}$ $p < 0.01$, comparison with medium control; ** $p < 0.01$, comparison with thrombin alone.

was assessed by quantitative real-time RT-PCR. Figure 1C shows that thrombin increases CCL2 mRNA levels within 30 min, with a maximal increase (21 ± 3-fold relative to control) observed at 6 h ($p < 0.01$). Thrombin-induced CCL2 protein release is completely blocked by actinomycin D (ActD; Figure 1E) at concentrations at which this transcriptional inhibitor also blocked the increase in thrombin-induced mRNA levels (Figure 1D). Thrombin-induced CCL2 protein release is therefore not due to the release of prestored CCL2.

The Stimulatory Effects of Thrombin on Fibroblast CCL2 Gene Expression and Protein Production Are Mediated via PAR_1 Coupling to $G\alpha_q$

To begin to unravel the mechanisms by which thrombin exerts its stimulatory effects on CCL2 mRNA and protein levels, we first examined the potential involvement of the high-affinity thrombin receptor PAR_1. Wild-type and PAR_1 knockout (KO) MLFs were exposed to thrombin (10 nM) and the specific PAR_1 agonist peptide TFLLR (200 μM) for 6 h (Figure 2A). Wild-type MLFs responded to thrombin and TFLLR, whereas PAR_1 KO MLFs were completely unresponsive (Figure 2A). The inactive reverse peptide RLLFT had no effect on either wild-type or PAR_1 KO MLFs. Experiments were also performed with TNF-α (10 ng/ml) as a positive control known to induce CCL2 independent of PAR signaling. The results show that both wild-type and PAR_1 KO MLFs respond normally to TNF-α (Figure 2A). Taken together, these data show that thrombin exerts its effects on CCL2 protein production and release via PAR_1 at this concentration of the proteinase.

PAR_1 exerts its pluripotent cellular effects via the ability to interact with multiple downstream G proteins, including $G\alpha_{i/o}$, $G\alpha_{q/11}$, and $G\alpha_{12/13}$. To identify the G protein involved in mediating PAR_1 activation–induced CCL2 release, we used minigene vectors that encode 11 unique carboxyl-terminal amino acid residues of the $G\alpha_q$, $G\alpha_i$, $G\alpha_{12}$, and $G\alpha_{13}$ subunits. These minigenes have previously been shown to effectively inhibit G protein signaling, including thrombin-mediated cellular effects (Ellis *et al.*, 1999; Gilchrist *et al.*, 2001; Vanhauwe *et al.*, 2002). Retroviral plasmids encoding EGFP or the C-terminal Gα minigenes were transduced into MLFs in parallel culture. Transduction efficiency was monitored by visualization of EGFP expression. Approximately 90% of EGFP-transduced cells were EGFP positive (data not shown). Figure 2B shows that the $G\alpha_q$ C-terminal antagonist encoding minigene completely abolished thrombin-induced CCL2 release. In contrast, transduction with a control minigene encoding EGFP, or C-terminal $G\alpha_i$, $G\alpha_{12}$, or $G\alpha_{13}$ had no effect on this response.

To further confirm the role of $G\alpha_q$ in this response, we examined the effect of a novel small molecule antagonist, Q94, which specifically targets PAR_1 coupling to $G\alpha_q$. Q94 has been shown to compete for binding at the carboxy terminus of activated PAR_1 with a high-affinity peptide mimic of carboxy terminal $G\alpha_q$, with an IC_{50} of 916 nM (see Supplementary Data Figure S1, left panel). Additionally, this

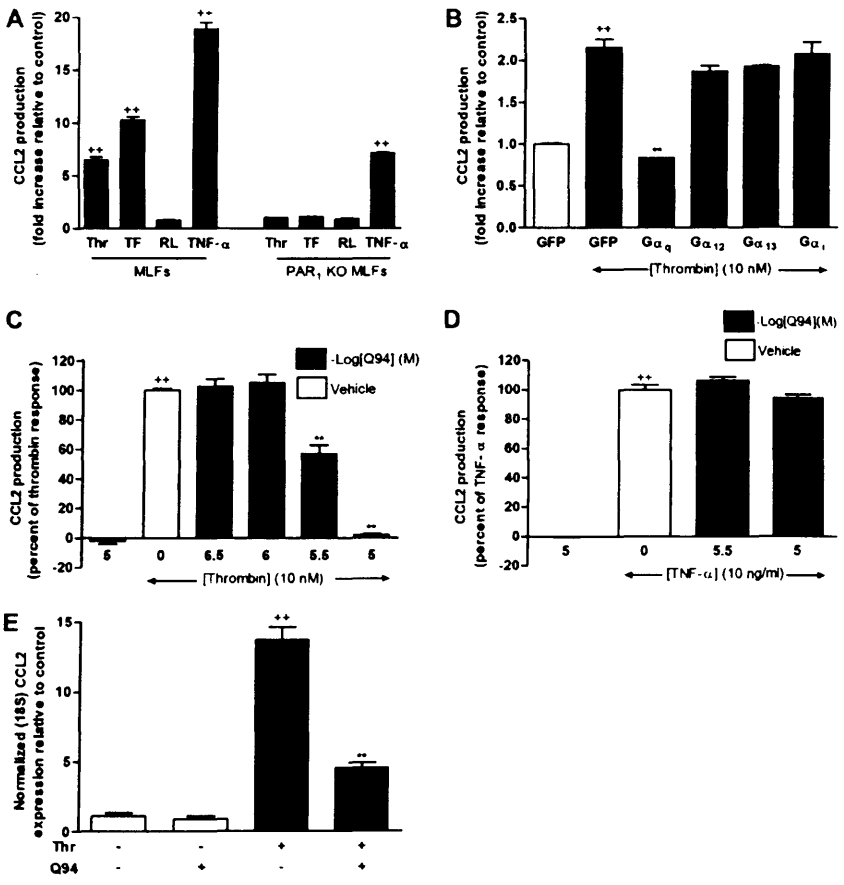

Figure 2. PAR_1 coupling to $G\alpha_q$ is necessary and sufficient for thrombin-induced CCL2 mRNA levels and protein release. (A) The effects of thrombin, PAR_1 agonist TFLLR-NH_2, the reverse peptide RLLFT-NH_2, and TNF-α on CCL2 protein release in MLFs and PAR_1 KO fibroblasts. Cells were exposed to thrombin (Thr, 10 nM), TFLLR-NH_2 (TF, 200 μM), RLLFT- NH_2 (RL, 200 μM), or TNF-α (10 ng/ml) for 6 h. CCL2 levels in culture supernatants were measured by ELISA. Data are presented as fold-increase relative to media control. (B) The effects of pRevTRE2-EGFP, pRevTRE2-$G\alpha_q$, pRevTRE2-$G\alpha_i$, pRevTRE2-$G\alpha_{12}$, or pRevTRE2-$G\alpha_{13}$ on thrombin-induced CCL2 protein release. MLFs transduced with the pRevTRE2-EGFP or the C-terminal Gα minigenes were exposed to thrombin (10 nM) for 6 h, and CCL2 levels in culture supernatant were measured by ELISA. Data are presented as fold change over control. (C and D) The effect of antagonist Q94 (targeting PAR_1 coupling to $G\alpha_q$) on thrombin- and TNF-α–induced CCL2 protein release. Data are presented as a percentage of the maximal response obtained with thrombin and drug vehicle alone (0.1% DMSO in DMEM). Cells were treated with increasing concentrations of Q94 for 3 h before exposure to thrombin for 6 h. Final concentrations of DMSO were kept constant for all treatment conditions. The first bar represents the highest dose of Q94 used and shows that this compound has no effect on basal CCL2 production. Negative log of the concentrations of Q94 are presented. (E) The effect of Q94 on thrombin-induced CCL2 mRNA levels. MLFs were exposed to thrombin for 2 h with or without preincubation with Q94 (10 μM) for 3 h. Data represent the mean ± SEM of triplicates. $^{++}p < 0.01$, comparison with medium control; and $^{**}p < 0.01$, comparison with thrombin alone.

compound has been shown to inhibit thrombin receptor activating peptide (TRAP) induced calcium transients in a concentration-dependent manner (see Supplementary Data Figure S1, right panel). Figure 2C shows that Q94 blocked PAR_1-mediated CCL2 production in a dose-dependent manner with complete inhibition obtained at 10 μM. Experiments performed with TNF-α as the stimulus showed that Q94 had no effect on TNF-α–stimulated CCL2 production (Figure 2D), indicating that the antagonist was specific for PAR_1 in blocking this response. We also examined the role of this antagonist on thrombin-induced CCL2 mRNA levels. Figure 2E shows that Q94 (10 μM) blocked thrombin-induced CCL2 mRNA levels by ~70% ($p < 0.01$). Taken together, these data show that $G\alpha_q$ plays a central role in PAR_1-induced CCL2 protein release and gene expression.

ERK1/2 Is Required for Thrombin-induced CCL2 Gene Expression and Protein Production

It has previously been reported that activation of $G\alpha_q$ by PAR_1 leads to the activation of ERK1/2 in MLFs (Trejo *et al.*, 1996). However, in terms of thrombin-induced CCL2 release by other cell types, the p38 MAPK pathway has been shown to play an important role (Brandes *et al.*, 2001; Marin *et al.*, 2001). Thus, we next performed experiments to determine the relative roles of the p38 MAPK and ERK1/2 pathways in PAR_1-induced CCL2 expression. Figure 3A shows that inhibition of p38 MAPK with SB203580 had no effect on thrombin-induced CCL2 protein release at concentrations at which this compound was effective at blocking thrombin-induced p38 phosphorylation (Figure 3B). It is worth pointing out that in these experiments, the inactive control compound SB202474 at the highest dose used significantly inhibited thrombin-induced CCL2 release by ~50% (data not shown), suggesting that at high concentrations these compounds may exert off-target effects. We therefore conclude that although thrombin activates p38 MAPK in MLFs, p38 is not involved in mediating PAR_1-induced CCL2 release. In contrast, preincubation of MLFs with the MEK1/2 inhibitor U0126 blocked this response in a dose-dependent manner from 0.3 μM onward (Figure 3C). The IC_{50} of U0126 was determined to be 1.3 μM for this response. U0126 also inhibited thrombin-induced phosphorylation of the MEK1/2 substrate, ERK1/2, in a dose-dependent manner (Figure 3D). We next determined the role of ERK1/2 in thrombin-induced CCL2 mRNA levels. Figure 3E shows that U0126 (1 μM) blocked the effect of thrombin on CCL2 mRNA levels by ~70% ($p < 0.01$).

To further examine the role of the ERK1/2 pathway in thrombin-induced CCL2 production, a genetic approach was used. Wildtype-(wt-MEK1), dominant-negative (dn-MEK1), or constitutively active (ca-MEK1) constructs were transduced into MLFs (Cowley *et al.*, 1994). Figure 3F shows that thrombin-induced ERK1/2 phosphorylation was blocked by up to 70% in cells transduced with dn-MEK1. Figure 3G shows that ca-MEK1 significantly increased basal ERK1/2 phosphorylation, but had no additive effect on thrombin-induced ERK1/2 phosphorylation. Transduction with dn-MEK1 also significantly reduced thrombin-induced CCL2 production, whereas transduction with ca-MEK1 significantly increased basal CCL2 production by about fourfold ($p < 0.01$), but had no additive effect on thrombin-stimulated CCL2 production (Figure 3H). These results provide strong evidence that the MEK1-ERK1/2 pathway rather than the p38 MAPK pathway is both necessary and sufficient for PAR_1-mediated CCL2 production in MLFs.

We next examined the role of PKC and c-Raf in thrombin-induced ERK1/2 activation and CCL2 expression. Both broad spectrum PKC inhibitors Ro-318425 (Figure 4A) and the c-Raf inhibitor (Figure 4B) blocked thrombin-induced ERK1/2 phosphorylation in a dose-dependent manner from 1 μM onward for Ro-318425 and 0.3 μM onward for c-Raf inhibitor. To determine whether PKC is upstream of c-Raf, cells were preincubated with Ro-318425, and c-Raf phosphorylation was assessed by Western blotting. As shown in Figure 4C, Ro-318425 (1 μM) completely blocked c-Raf phosphorylation induced by thrombin.

We next determined whether PKC and c-Raf are involved in thrombin-induced CCL2 production and gene expression. Figure 4, D–F, shows that both PKC broad spectrum inhibitors Ro-318425 and GF109203X, and the c-Raf inhibitor inhibited thrombin-induced CCL2 release in a dose-dependent manner. At concentrations of Ro-318425 and c-Raf inhibitor that blocked thrombin-induced ERK1/2 phosphorylation, thrombin-induced CCL2 production was blocked by 56 ± 4% ($p < 0.01$) with Ro-318425 (1 μM) and by 61 ± 5% ($p < 0.01$) with the c-Raf inhibitor (3 μM). At these concentrations, these inhibitors inhibited thrombin-induced CCL2 mRNA accumulation by 75 and 81%, respectively (Figure 4, F and G). Taken together, these data show that PKC, c-Raf, and ERK1/2 are in a linear pathway for thrombin-induced CCL2 gene expression and protein production.

The Rho Kinase Pathway Mediates Thrombin-induced CCL2 Protein Release via a Nontranscriptional Mechanism

The data obtained so far point to a central role for $G\alpha_q$ and ERK1/2 in mediating the effects of PAR_1 activation on CCL2 production. Although, $G\alpha_{12/13}$ is generally considered as a major activator of Rho kinase, there is emerging evidence that $G\alpha_q$ is also able to signal via Rho kinase by activating the PLC-Ca^{2+} pathway (Singh *et al.*, 2007). Moreover, thrombin is known to mediate Ca^{2+} signaling via PAR_1 in fibroblasts, and this can be inhibited by BAPTA (Trejo *et al.*, 1996; Tanaka *et al.*, 2004). We therefore next examined the role of the PLC-Ca^{2+} pathway and Rho kinase in PAR_1-mediated CCL2 release. Cells were treated with increasing concentrations of U73122 (PLC inhibitor, Figure 5A), BAPTA-AM (Ca^{2+} chelator, Figure 5B), Gö6976 (Ca^{2+}-dependent PKC inhibitor, Figure 5C), or Y-27632 and H-1152 (Rho kinase inhibitors, Figure 5, D and E). The data obtained show that all these inhibitors blocked PAR_1-mediated CCL2 production in a dose-dependent manner. The IC_{50} of each inhibitor was determined to be 1.5 μM for U73122, 1 μM for BAPTA-AM, 0.5 μM for Gö6976, 3 μM for Y-27632, and 2.7 μM for H-1152, for this response. In contrast, the inactive control compound for U73122, U73343, was found to have no effect on PAR_1-mediated CCL2 production at the highest concentrations used (data not shown).

To determine whether these kinases are upstream of the ERK1/2 pathway, we examined the effects of these inhibitors at their respective IC_{50} concentrations on thrombin-induced ERK1/2 phosphorylation. Figure 5F shows that none of the four inhibitors interfered with thrombin-induced ERK1/2 phosphorylation, indicating that PLC, Ca^{2+}, Ca^{2+}-dependent PKC, and Rho kinase mediate their effects on thrombin-induced CCL2 protein release in an ERK1/2-independent manner.

To determine whether PLC, Ca^{2+}, and Ca^{2+}-dependent PKC signal via Rho kinase, we examined the effects of the above inhibitors on the phosphorylation of myosin light chain (MLC), a downstream substrate of Rho kinase (Amano *et al.*, 1996). Figure 5G shows that PAR_1 activation induces MLC phosphorylation within 10 min of stimulation and that this response is blocked by U73122, BAPTA-AM, Gö679, and

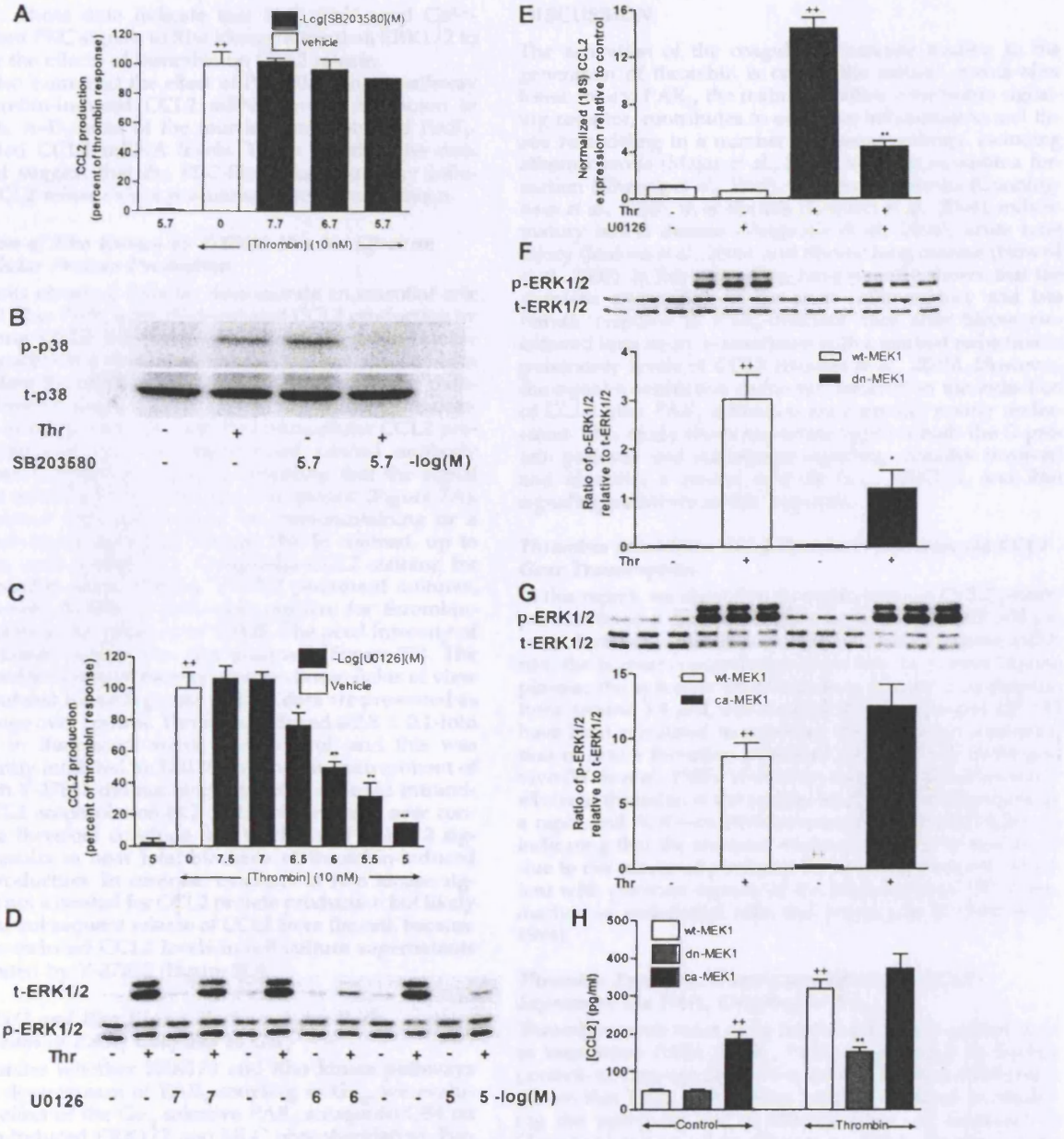

Figure 3. ERK1/2 is necessary and sufficient for thrombin-induced CCL2 mRNA levels and protein release. (A) The effect of SB203580 on CCL2 protein release in response to thrombin. Data are presented as a percentage of the maximal response obtained with thrombin and drug vehicle alone (0.2% DMSO in DMEM). Cells were treated with increasing concentrations of SB203580 for 30 min before exposure to thrombin (10 nM) for 6 h. Final concentrations of DMSO were kept constant for all treatment conditions. The first bar represents the highest dose of SB203580 examined and shows that this compound has no effect on basal CCL2 production. Negative log of the concentration of SB203580 is presented. (B) The effect of SB203580 (2 μM) on thrombin-induced p38 phosphorylation. Cells were treated with or without SB203580 for 30 min before exposure to thrombin for 2 min. p38 phosphorylation was assessed by Western blotting of cell lysates using an anti-phospho-p38 antibody (B, p-p38). Protein loading was verified by blotting with an anti-p38 antibody (B, t-p38). The blot is representative of three separate experiments performed. (C) The effect of U0126 on CCL2 protein release in response to thrombin. Data were analyzed as in A. (D) Dose–response data for the effect of U0126 on thrombin-induced ERK1/2 phosphorylation. Cells were treated with increasing concentrations of U0126 for 30 min before exposure to thrombin for 2 min. ERK1/2 phosphorylation was assessed by Western blotting of cell lysates as in B. (E) The effect of U0126 on thrombin-induced CCL2 mRNA levels. MLFs were exposed to thrombin (10 nM) for 2 h with or without preincubation with U0126 (1 μM) for 30 min. CCL2 mRNA levels were determined by quantitative RT-PCR. (F and G) The effects of wt-MEK1, dn-MEK1, or ca-MEK1 on thrombin-induced ERK1/2 phosphorylation. MLFs transduced with MEK1-pBabePuro constructs expressing the wt-MEK1, dn-MEK1 (F) or ca-MEK1 (G) were exposed to thrombin (10 nM) for 2 min. ERK1/2 phosphorylation was assessed by Western blotting of cell lysates using an anti-phospho-ERK1/2 antibody (F and G, p-ERK1/2). Protein loading was verified by blotting with an anti-ERK1/2 antibody (F and G, t-ERK1/2). The blot is representative of three separate experiments performed. The column graphs represent the densitometry analysis of the blots as a result of phospho-ERK1/2 relative to total ERK1/2 and are representative of three separate

Y-27632. These data indicate that PLC, Ca^{2+}, and Ca^{2+}-dependent PKC signals to Rho kinase rather than ERK1/2 to mediate the effects of thrombin on CCL2 release.

We also examined the effect of PLC-Rho kinase pathway on thrombin-induced CCL2 mRNA levels. As shown in Figure 6, A–D, none of the four inhibitors blocked PAR_1-stimulated CCL2 mRNA levels. Taken together the data obtained suggest that the PLC-Rho kinase pathway influences CCL2 release via a posttranscriptional mechanism.

Inhibition of Rho Kinase by Y-27632 Has No Effect on Intracellular Protein Production

The results obtained thus far demonstrate an essential role for ERK1/2 in PAR_1 activation-induced CCL2 production by influencing CCL2 mRNA levels, whereas the Rho kinase pathway acts via a posttranscriptional mechanism. To begin to elucidate the mechanism by which the Rho kinase pathway influences this response, we used immunocytofluorescence to examine thrombin-induced intracellular CCL2 protein production. The isotype-matched control antibody panels are completely negative, indicating that the signal obtained with the CCL2 antibody was specific (Figure 7A). Unstimulated cells also yielded no immunostaining or a very weak signal for CCL2 (Figure 7B). In contrast, up to 60% cells were positive for intracellular CCL2 staining for both thrombin alone and for Y-27632 pretreated cultures, whereas only 5–10% of cells were positive for thrombin-treated cells in the presence of U0126. The pixel intensity of each treatment group was also analyzed (Figure 7F). The mean pixel intensity of three randomly chosen fields of view was calculated for each group, and the data are presented as fold change over control. Thrombin induced a 2.8 ± 0.1-fold increase in fluorescent signal over control, and this was significantly inhibited by U0126. In contrast, pretreatment of cells with Y-27632 did not block thrombin-induced intracellular CCL2 accumulation (3.2 ± 0.5-fold increase over control). We therefore conclude that blockade of ERK1/2 signaling results in near total blockade of thrombin-induced CCL2 production. In contrast, blockade of Rho kinase signaling is not essential for CCL2 protein production but likely affects the subsequent release of CCL2 from the cell, because thrombin-induced CCL2 levels in cell culture supernatants are inhibited by Y-27632 (Figure 5D).

The ERK1/2 and Rho Kinase Pathways Are Both Downstream of PAR_1 Coupling to $G\alpha_q$

To determine whether ERK1/2 and Rho kinase pathways are both downstream of PAR_1-coupling to $G\alpha_q$, we evaluated the effect of the $G\alpha_q$ selective PAR_1 antagonist Q94 on thrombin-induced ERK1/2 and MLC phosphorylation. Figure 8 shows that Q94 completely blocked thrombin-induced ERK1/2 (Figure 8A) and MLC phosphorylation (Figure 8B). These data confirm that ERK1/2 and Rho kinase pathways are downstream of PAR_1-coupling to $G\alpha_q$ and act in cooperation to mediate the effects of PAR_1 activation on CCL2 gene expression, protein production and release.

Figure 3 (cont). experiments performed with three replicates per sample. (H) The effects of wt-MEK1, dn-MEK1, or ca-MEK1 on thrombin-induced CCL2 protein release. MLFs transduced with MEK1-pBabePuro constructs expressing the wt-MEK1, dn-MEK1, or ca-MEK1 were exposed to thrombin (10 nM) for 6 h, and subsequent CCL2 protein levels in culture supernatants were measured by ELISA. $^{**}p < 0.01$, comparison with medium control; $^{*}p < 0.05$ and $^{**}p < 0.01$, comparison with thrombin alone.

DISCUSSION

The activation of the coagulation cascade leading to the generation of thrombin is one of the earliest events after tissue injury. PAR_1, the main high-affinity thrombin signaling receptor, contributes to excessive inflammation and tissue remodeling in a number of disease settings, including atherosclerosis (Major *et al.*, 2003), vascular neointima formation (Cheung *et al.*, 1999), glomerulonephritis (Cunningham *et al.*, 2000), liver fibrosis (Fiorucci *et al.*, 2004), inflammatory bowel disease (Vergnolle *et al.*, 2004), acute lung injury (Jenkins *et al.*, 2006), and fibrotic lung disease (Howell *et al.*, 2005). In this setting, we have recently shown that the dramatic attenuation of the acute inflammatory and late fibrotic response in PAR_1-deficient mice after bleomycin-induced lung injury is associated with a marked reduction in pulmonary levels of CCL2 (Howell *et al.*, 2005). However, the signal transduction pathways involved in the induction of CCL2 after PAR_1 activation are currently poorly understood. This study sheds important light on both the G protein pathway and subsequent signaling cascades involved and identifies a central role for $G\alpha_q$, ERK1/2, and Rho signaling pathways in this response.

Thrombin Stimulates CCL2 Protein Production via CCL2 Gene Transcription

In this report, we show that thrombin induces CCL2 protein production in a dose-dependent manner from 0.03 nM onward. Strikingly, this response did not reach a plateau at 300 nM, the highest concentration examined. In normal human plasma, the zymogen prothrombin is present at concentrations around 1.4 μM, but concentrations of around 130 nM have been calculated to represent the maximum concentration of active thrombin generated during blood clotting in vivo (Walz *et al.*, 1985). We further show that the stimulatory effects of thrombin at the protein level were accompanied by a rapid and ActD-sensitive increase in CCL2 mRNA levels, indicating that the observed increase in CCL2 release is not due to the release of prestored CCL2. These data are consistent with previous reports of thrombin-induced CCL2 production in endothelial cells and monocytes (Colotta *et al.*, 1994).

Thrombin Exerts Its Stimulatory Effects on CCL2 Expression via PAR_1 Coupling to $G\alpha_q$

Thrombin exerts most of its cellular effects via activation of at least three PARs (PAR_1, PAR_3, and PAR_4) by limited proteolytic cleavage of the N-terminus. We and others have shown that PAR_1 is the major receptor involved in mediating the mitogenic and profibrotic effects of thrombin in fibroblasts (reviewed in Chambers, 2003). In the present study, the involvement of PAR_1 in mediating CCL2 production was demonstrated in experiments employing the highly selective PAR_1 peptide agonist, $TFLLR\text{-}NH_2$. This agonist activates PAR_1 independently of receptor cleavage, and unlike the commonly used peptide agonists, based on the tethered ligand sequence of PAR_1 (SFLLRN), does not activate PAR_2 in human mesenchymal cells (Hollenberg *et al.*, 1997). The necessity for PAR_1 in mediating the effects of thrombin at 10 nM was confirmed in experiments demonstrating that CCL2 production was not up-regulated in fibroblasts derived from PAR_1 KO mice.

Although many of the resultant biological consequences of PAR_1 activation in fibroblasts are known, less is known about which G proteins mediate these events. The C-terminal region of Gα subunits has been shown to be critical in determining the specificity of GPCR–G protein interactions

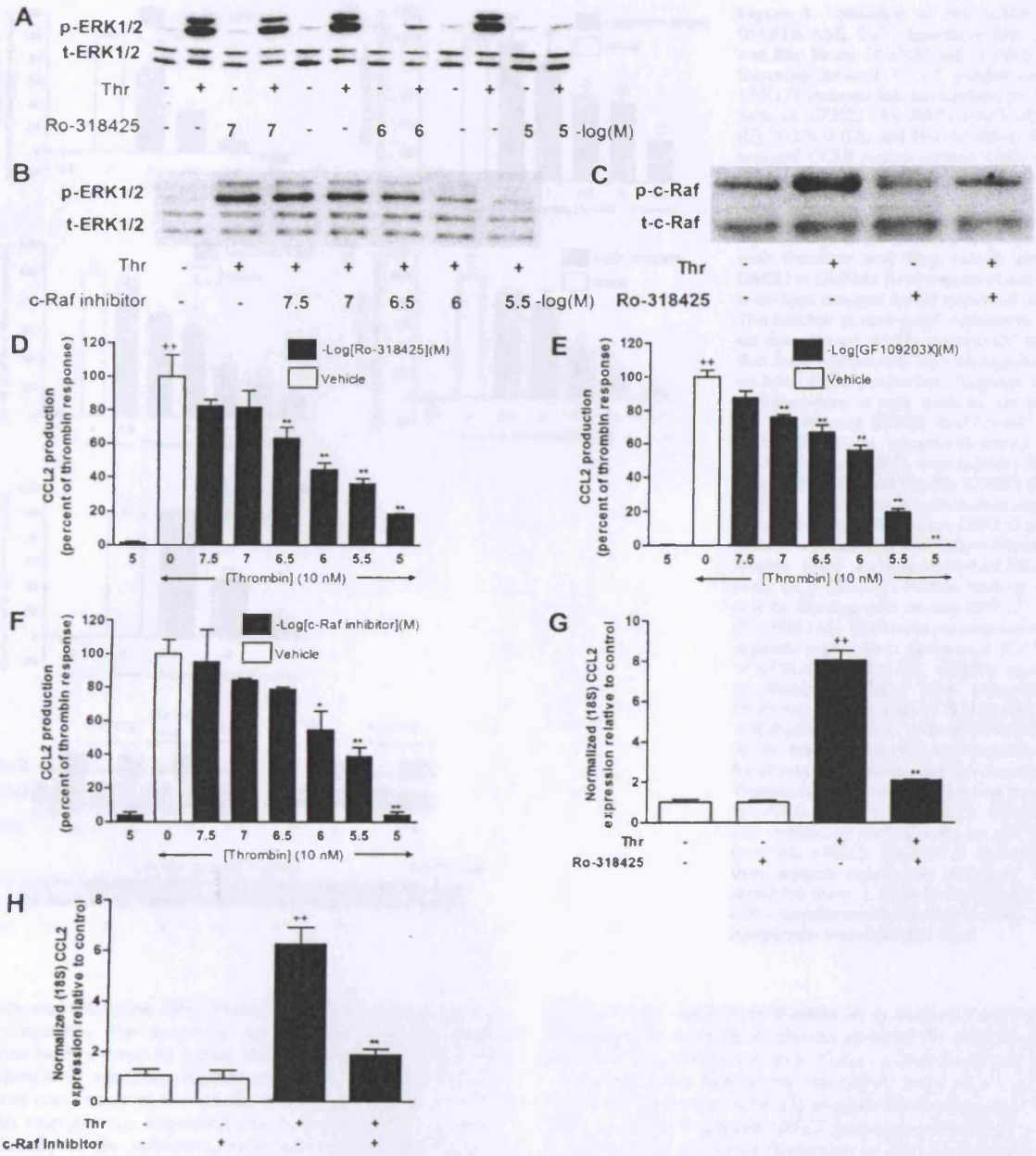

Figure 4. Inhibition of c-Raf kinase and PKC attenuates thrombin-induced ERK1/2 phosphorylation, CCL2 protein production, and CCL2 mRNA levels. (A and B) Dose–response data for the effects of Ro-318425 (A) and c-Raf inhibitor (B) on thrombin-induced ERK1/2 phosphorylation. Cells were treated with increasing concentrations of inhibitors for 30 min before exposure to thrombin (10 nM) for 2 min. ERK1/2 phosphorylation was assessed by Western blotting of cell lysates using an anti-phospho-ERK1/2 antibody (A and B, p-ERK1/2). Protein loading control was verified by blotting with an anti-ERK1/2 antibody (A and B, t-ERK). The blots are representative of three separate experiments performed. (C) The effect of Ro-318425 on thrombin-induced c-Raf phosphorylation. Cells were treated with Ro-318425 (1 μM) for 30 min before stimulation with thrombin (10 nM) for 10 min. c-Raf phosphorylation was assessed by Western blotting of cell lysates using an anti-phospho-c-Raf antibody (C, p-c-Raf). Protein loading was verified by blotting with an anti-c-Raf antibody (C, p-c-Raf). The blot is representative of three separate experiments performed. (D–F) The effects of Ro-318425 (D), GF109203X (E), and c-Raf inhibitor (F) on CCL2 protein production in response to thrombin. Data are presented as a percentage of the maximal response obtained with thrombin (10 nM) and drug vehicle alone (0.1% DMSO in DMEM). Cells were treated with increasing concentrations of Ro-318425, GF109203X, or c-Raf kinase inhibitor for 30 min before exposure to thrombin for 6 h. Final concentrations of DMSO were kept constant for all treatment conditions. The first bar in each graph represents the highest doses of inhibitors examined and shows that these compounds have no effect on basal CCL2 production. Negative log of the concentrations of inhibitors are presented. (G and H) The effects of Ro-318425 (G) and c-Raf inhibitor (H) on thrombin-induced CCL2 mRNA levels. MLFs were exposed to thrombin (10 nM) for 2 h with or without preincubation with c-Raf inhibitor (3 μM) or Ro-318425 (1 μM) for 30 min. CCL2 mRNA levels were determined by quantitative RT-PCR. Final concentrations of DMSO were kept constant for all treatment conditions (0.01% DMSO in DMEM). Data represent the mean ± SEM from triplicates. $^{++}$ $p < 0.01$, comparison with medium control; ** $p < 0.01$, comparison with thrombin alone.

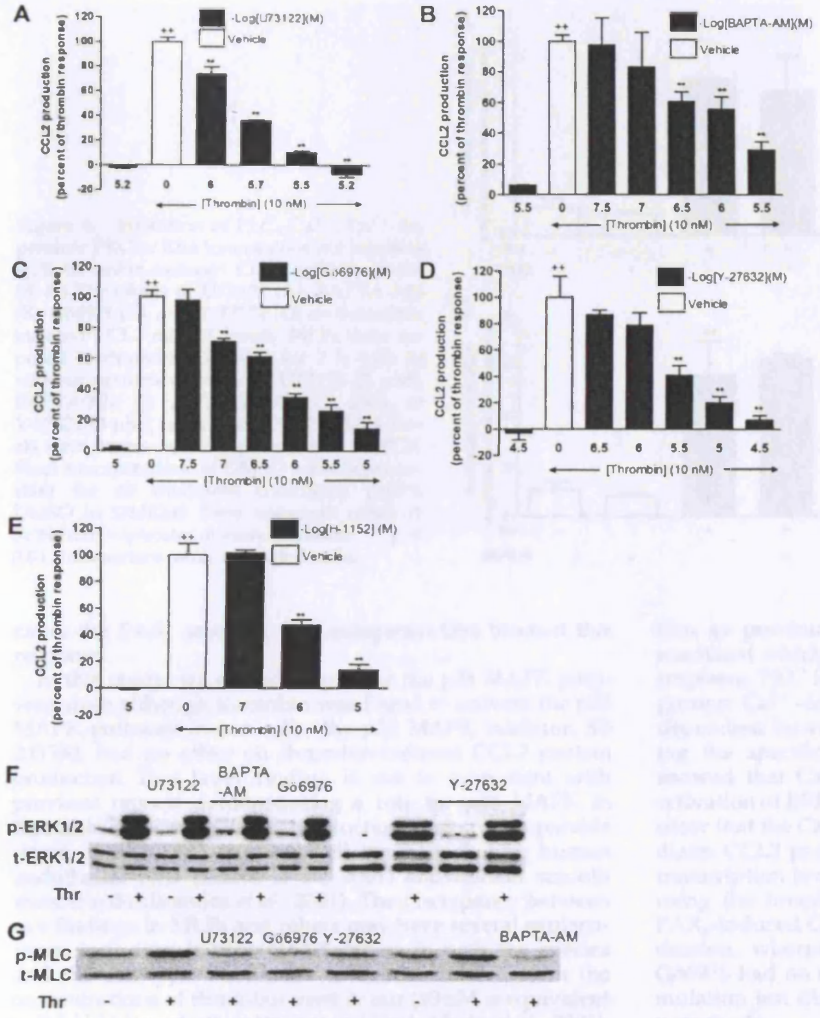

Figure 5. Inhibition of PLC (U73122), Ca^{2+} (BAPTA-AM), Ca^{2+}-dependent PKC (Gö6976), and Rho kinase (Y-27632 and H-1152) attenuate thrombin-induced CCL2 production in an ERK1/2-independent mechanism. (A–E) The effects of U73122 (A), BAPTA-AM (B), Gö6976 (C), Y-27632 (D), and H-1152 (E) on thrombin-induced CCL2 protein release. Cells were preincubated with increasing concentrations of each inhibitor for 30 min before exposure to thrombin (10 nM) for 6 h. Data are presented as a percentage of the maximal response obtained with thrombin and drug vehicle alone (0.1% DMSO in DMEM). Final concentrations of DMSO were kept constant for all treatment conditions. The first bar in each graph represents the highest dose of each inhibitor examined and shows that these compounds have no significant effect on basal CCL2 production. Negative log of the concentrations of each inhibitor are presented. (F) The effect of U73122, BAPTA-AM, Gö6976, and Y-27632 on thrombin-induced ERK1/2 phosphorylation. MLFs were treated with U73122 (2 μM), BAPTA-AM (2 μM), Gö6976 (1 μM), or Y-27632 (3 μM) for 30 min before exposure to thrombin (10 nM) for 2 min. ERK1/2 phosphorylation was assessed by Western blotting of cell lysates using an anti-phospho-ERK1/2 antibody (F, p-ERK1/2). Protein loading was verified by blotting with an anti-ERK1/2 antibody (F, t-ERK1/2). The blot is representative of three separate experiments performed. (G) The effect of U73122, BAPTA-AM, Gö6976, and Y-27632 on thrombin-induced MLC phosphorylation. MLFs were treated with U73122 (2 μM), BAPTA-AM (2 μM), Gö6976 (1 μM), or Y-27632 (3 μM) for 30 min before stimulation with thrombin (10 nM) for 10 min. MLC phosphorylation was assessed by Western blotting of cell lysates using an anti-phospho-MLC antibody (G, p-MLC). Protein loading was verified by blotting with an anti-MLC antibody (G, t-MLC). The blot is representative of three separate experiments performed. Data represent the mean ± SEM from triplicates. $^{++}p < 0.01$, comparison with medium control; $^{**}p < 0.01$, comparison with thrombin alone.

(Hamm and Gilchrist, 1996; Hamm, 1998). This interaction is highly specific. For example, substituting a single amino acid has been shown to annul the ability of $G\alpha_i$ to bind the A_1 adenosine receptor (Gilchrist *et al.*, 1998). Dominant-negative constructs of the α subunit of G proteins (termed G protein minigenes), encoding the C-terminal sequence for each family of $G\alpha$ subunits, have been developed as powerful tools for identifying the G protein that mediates a given physiological function after thrombin activation (Gilchrist *et al.*, 2001). In experiments employing such a $G\alpha$ subunit minigene approach, we were able to show that only $G\alpha_q$ is necessary for PAR_1-mediated CCL2 protein release, and that $G\alpha_{12}$, $G\alpha_{13}$, and $G_{\alpha i}$ play no role. The critical involvement of $G\alpha_q$ was further confirmed with a recently developed novel PAR_1 selective $G\alpha_q$ protein signaling antagonist (Q94, Caden Biosciences), which blocked PAR_1-mediated increases in both CCL2 mRNA and protein levels in a dose-dependent manner. It is worth mentioning that in these experiments, the CCL2 response obtained was usually greater for TFLLR-NH_2 over thrombin. This observation might be explained by recent evidence that there are differences between thrombin and peptide agonists in terms of the subsequent ability of PAR_1 to activate different G proteins (McLaughlin *et al.*, 2005). PAR_1 activation studies in endothelial cells showed that peptide activation altered receptor/G protein binding to favor $G\alpha_q$ activation over $G\alpha_{12/13}$. It is tempting to speculate that such functional selectivity may also occur in fibroblasts and may similarly explain the finding that TFLLR-NH_2 induces a greater CCL2 response compared with the physiological activator thrombin in our experiments.

PAR_1 Activation Induces CCL2 Release via a Ca^{2+}-dependent PKC/ERK1/2 Pathway and Increased CCL2 Gene Expression

$G\alpha_q$ has been shown to be necessary for mediating thrombin-induced ERK1/2 phosphorylation in MLFs (Trejo *et al.*, 1996). We therefore examined the possibility that the ERK1/2 pathway may be downstream of PAR_1 coupling to $G\alpha_q$ and therefore may be involved in thrombin-induced CCL2 gene expression and protein production. Using either the pharmacological inhibitor, U0126, or dn-MEK1 gene constructs, we show that the ERK1/2 pathway is necessary for CCL2 protein production and release mediated by PAR_1 coupling to $G\alpha_q$. $G\alpha_q$ was further found to be necessary for mediating thrombin-induced ERK1/2 phosphorylation be-

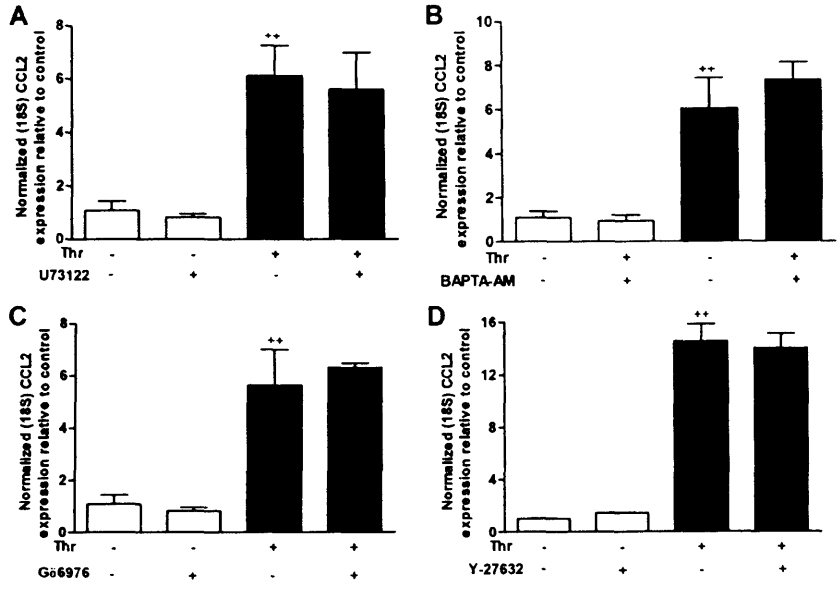

Figure 6. Inhibition of PLC, Ca^{2+}, Ca^{2+}-dependent PKC or Rho kinase does not interfere with thrombin-induced CCL2 mRNA levels. (A–D) The effects of U73122 (A), BAPTA-AM (B), Gö6976 (C), and Y-27632 (D) on thrombin-induced CCL2 mRNA levels. MLFs were exposed to thrombin (10 nM) for 2 h with or without preincubation with U73122 (2 μM), BAPTA-AM (2 μM), Gö6976 (1 μM), or Y-27632 (3 μM) for 30 min. CCL2 mRNA levels were determined by quantitative RT-PCR. Final concentrations of DMSO were kept constant for all treatment conditions (0.01% DMSO in DMEM). Data represent mean ± SEM from triplicates at each condition. $^{++}$ $p < 0.01$, comparison with untreated cells.

cause the PAR_1 selective $G\alpha_q$ antagonist Q94 blocked this response.

In this study, we exclude a role for the p38 MAPK pathway since although thrombin was found to activate the p38 MAPK pathway in our cells, the p38 MAPK inhibitor, SB 203580, had no effect on thrombin-induced CCL2 protein production This latter finding is not in agreement with previous reports demonstrating a role for p38 MAPK in thrombin-induced CCL2 production using comparable doses of SB203580 in other cell types, including human endothelial cells (Marin *et al.*, 2001) and human smooth muscle cells (Brandes *et al.*, 2001). The discrepancy between our findings in MLFs and others may have several explanations, including important differences in both the species and the cell type examined, as well as differences in the concentrations of thrombin used in our (10 nM is equivalent to 0.5 U/ml) and other studies (8 U/ml; Marin *et al.*, 2001). Moreover, from our experience with PAR_1 KO fibroblasts, we are aware that thrombin can induce CCL2 release at higher concentrations via a non-PAR mediated mechanism because only PAR_1 and PAR_4 are expressed, and PAR_4 agonist peptides fail to induce CCL2 release in PAR_1 KO fibroblasts (data not shown). As thrombin is a proteinase, we propose it is likely that at higher concentrations, CCL2 protein production may be mediated via the release of other stimulatory mediators bound to the pericellular matrix. In this regard, thrombin has previously been shown to release TGF-β bound to the pericellular matrix of fibroblasts (Taipale *et al.*, 1992). It is therefore possible that non-PAR_1–dependent pathways lead to activation of the p38 MAPK pathway to mediate thrombin-induced CCL2 release at high concentrations of the proteinase. Although concentrations around 130 nM may be generated after intravascular coagulation and are therefore relevant in the context of thrombin signaling in vascular cell types, thrombin concentrations in extravascular compartments and hence in the context of fibroblast signaling responses are likely to be much lower.

The downstream signaling molecules that mediate the actions of G proteins on ERK1/2 include ras, PKC, and c-Raf kinase (Blumer and Johnson, 1994), which can activate MEK (Cobb *et al.*, 1994). Indeed, our data show that both c-Raf and PKC are involved in PAR_1-mediated ERK1/2 phosphorylation as previously reported (Trejo *et al.*, 1996). We also examined which class of PKC isozymes are involved in this response. PKC isozymes can be generally divided into two groups: Ca^{2+}-dependent (conventional PKC) and Ca^{2+}-independent (novel and atypical isozymes). Experiments using the specific Ca^{2+}-dependent PKC inhibitor, Gö6976, showed that Ca^{2+}-dependent PKC is not involved in the activation of ERK1/2 after PAR_1 activation. Our data further show that the Ca^{2+}-independent PKC-ERK1/2 pathway mediates CCL2 protein production by influencing CCL2 gene transcription because inhibition of ERK1/2, c-Raf, and PKC using the broad-spectrum inhibitor, Ro-318425, abolished PAR_1-induced CCL2 mRNA accumulation and protein production, whereas inhibition of Ca^{2+}-dependent PKC by Gö6976 had no effect on PAR_1-induced CCL2 mRNA accumulation but did inhibit CCL2 release into cell culture supernatants.

The Ca^{2+}-dependent PKC Rho Pathway Mediates the Effects of PAR_1 Activation on CCL2 Release via a Posttranscriptional Mechanism

Ca^{2+}-dependent PKC is a well-recognized downstream effector of $G\alpha_q$ and is activated in response to a rise in intracellular Ca^{2+} concentration. Although the α-subunit of $G\alpha_q$ is well-known to trigger an increase in intracellular Ca^{2+} concentration by stimulating PLC-β activity and $G\alpha_{12/13}$ activation preferentially induces Rho kinase activation, a recent study by Singh *et al.* (2007) demonstrated that $G\alpha_q$ can lead to Rho kinase activation via PLC-β-Ca^{2+}–dependent PKC to mediate thrombin-induced endothelial cell contraction. In the present study, our data similarly suggest that Rho kinase activation is mediated by a $G\alpha_q$-PLC-Ca^{2+}–dependent PKC pathway to mediate thrombin-induced CCL2 release. First, we show that inhibitors of PLC, Ca^{2+}, and Ca^{2+}-dependent PKC all block thrombin-induced phosphorylation of MLC, one of the major downstream substrates of Rho kinase (Amano *et al.*, 1996). Second, a direct link between this pathway and $G\alpha_q$ coupling to PAR_1 was demonstrated by showing that the novel PAR_1 selective $G\alpha_q$ antagonist (Q94) also abolishes thrombin-induced MLC phosphorylation.

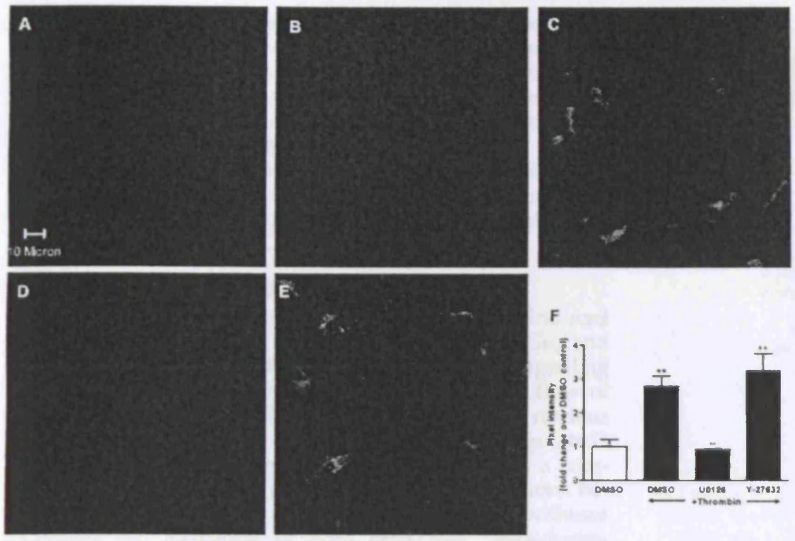

Figure 7. Immunocytofluorescence demonstrates that CCL2 intracellular protein production is blocked by MEK1/2 inhibition (U0126) but not by Rho kinase inhibition (Y-27632). (A) Isotype control; (B) untreated cells; (C) the effect of thrombin on CCL2 intracellular protein production; (D) the effect of U0126 on CCL2 intracellular protein production induced by thrombin; (E) the effect of Y-27632 on CCL2 intracellular protein production induced by thrombin. Cells were preincubated with or without U0126 (10 μM) or Y-27632 (10 μM) for 30 min before stimulation with thrombin (10 nM) for 3 h. At the end of incubation, cells were immunostained with normal goat IgG (A) or anti-CCL2 antibody (B–E) followed by DAPI staining. Areas of CCL2 localization are shown in green, and nuclei appear blue. (F) The mean pixel intensity of three randomly chosen fields of view, expressed as fold increase over DMSO control, for each group. $^{++}$ $p < 0.01$, comparison with untreated cells. ** $p < 0.01$, comparison with thrombin alone.

Our findings and those reported by Singh *et al.* (2007) contrast with a previous report demonstrating that $G\alpha_q$-mediated Rho kinase activation occurs independently of PLCβ in mouse embryonic fibroblasts (MEFs; Vogt *et al.*, 2003). The activation of Rho A requires its dissociation from the Rho kinase/GDP/GDI-1 complex followed by GTP exchange mediated by guanine nucleotide exchange factors (GEFs). In terms of Rho kinase activation by GPCRs, the RhoGEF (Rho guanine nucleotide exchange factor) proteins, p115RhoGEF, PDZ-RhoGEF, and leukemia-associated Rho guanine-nucleotide exchange factor (LARG), have been shown to stimulate Rho kinase activity (Hart *et al.*, 1998; Kozasa *et al.*, 1998; Fukuhara *et al.*, 2001; Suzuki *et al.*, 2003). Singh and colleagues provided evidence that $G\alpha_q$-mediated Rho kinase activation is p115RhoGEF dependent after PLC-Ca^{2+}–dependent PKC activation, whereas in studies by Vogt and colleagues in MEFs, thrombin-induced Rho kinase activation was shown to be mediated by the RhoGEF, LARG. Taken together, our findings are consistent with a PLC-Ca^{2+}–dependent PKC-dependent Rho kinase activation mechanism rather than a LARG-mediated mechanism.

Although Rho kinase was first characterized as a major regulator of actin dynamics, it is now well recognized that Rho family proteins influence a range of other cellular processes, including gene transcription and protein secretion (reviewed in Etienne-Manneville and Hall, 2002). In the present study, we provide evidence that the Rho kinase pathway is involved in PAR_1-mediated CCL2 protein release posttranscriptionally, likely by influencing protein secretion. This is based on two observations. First, inhibition of Rho kinase only blocks thrombin-induced CCL2 protein release but not CCL2 mRNA levels. Second, immunocytofluorescence experiments to elucidate the mechanism by which the Rho kinase pathway influences CCL2 release, demonstrate that intracellular protein production is blocked with the MEK1/2 inhibitor, U0126, but is unaffected by the Rho kinase inhibitor, Y-27632. Taken together, these data indicate that blockade of Rho kinase signaling is not essential for PAR_1-mediated CCL2 gene expression and protein production. Given that Y-27632 blocks PAR_1-mediated increases in CCL2 levels measured in cell culture supernatants, we propose that this pathway most likely affects CCL2 protein secretion.

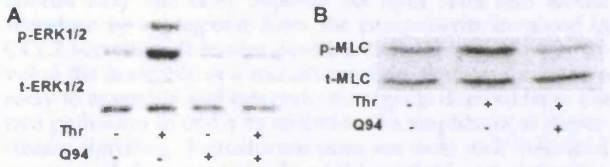

Figure 8. ERK1/2 and MLC pathways are downstream of PAR_1 coupling to $G\alpha_q$. (A and B) The effect of Q94 on thrombin-induced ERK1/2 (A) and MLC (B) phosphorylation. Cells were treated with Q94 (10 μM) for 3 h before exposure to thrombin (2 min for ERK1/2, 10 min for MLC). Phosphorylation of ERK1/2 or MLC was assessed by Western blotting of cell lysates using an anti-phospho-ERK1/2 antibody (A, p-ERK1/2) or an anti-phospho-MLC antibody (B, p-MLC). Protein loading was verified by blotting with an anti-ERK1/2 antibody (A, t-ERK1/2) or an anti-MLC antibody (B, t-MLC). The blots are representative of three separate experiments.

Specificity of Pharmacological Inhibitors Used

In this study we used a range of small cell-permeable inhibitors of protein kinases to dissect the signaling pathways involved in PAR_1-mediated CCL2 release. These inhibitors exhibit a relatively high degree of specificity for the target kinase, but these compounds are not always totally specific and need to be used with caution. In this study, all experiments involving such inhibitors were designed according to expert recommendations (Davies *et al.*, 2000; Bain *et al.*, 2003). First, the role of most of the targeting kinases was verified by more than one structurally unrelated inhibitor. For instance, the involvement of ERK1/2 was confirmed by both U0126 and PD98059 (data no shown). Similarly for PKC, we used the broad-spectrum PKC inhibitors, Ro-318425 and GF109203X. For Rho kinase, we used two compounds: Y-27632 and H-1152. Second, wherever available, inactive control compounds (e.g., SB202474 as a negative control for SB203580 and U73343 as a negative control for U73122) for each inhibitor were tested in parallel cultures. This was particularly important when high concentrations

of the active compounds were used. Third, genetic approaches were used to strengthen the results obtained with MEK1/2 inhibition and to identify the G protein involved in coupling to PAR_1. Finally, we demonstrated that most inhibitors blocked CCL2 release at the same concentrations that prevented the phosphorylation of an authentic physiological substrate of the protein kinase. For example, ERK1/2, p38 MAPK, and MLC phosphorylation was assessed to identify the lowest concentration of respective inhibitors required for subsequent CCL2 release experiments.

Integration of the Flow of Information

In this study, we provide evidence that thrombin-induced CCL2 release is mediated via coupling of PAR_1 to $G\alpha_q$ and the cooperation between ERK1/2 and Rho kinase signaling pathways. This raises the question as to how the flow of information to the two pathways, one directed at the nucleus and the other at a specific secretory apparatus, is regulated? Signal transduction leading to cellular responses is a complex processes initiated by protein–protein interactions between ligands, receptors, and kinases. Recent hypotheses including the formation of lipid rafts and "transduceosomes" have shed light on how these multiple components work in harmony. Lipid rafts are specialized structures on the plasma membrane that have an altered lipid composition as well as links to the cytoskeleton. Recent studies indicate that some GPCRs, G proteins, and their effectors localize to lipid rafts or dynamically move in and out of microdomains (Simons and Toomre, 2000). A recent study has shown that PAR_1 is present in endothelial cell plasma membrane rafts and caveolae and that the localization of PAR_1 specifically to rafts serves as an important mechanism for the regulation of thrombin-induced cytoskeletal changes in endothelial cells. Of particular interest to the current work, these authors further demonstrate a role for lipid rafts in mediating PAR_1 activation of both RhoA/Rho kinase signaling and MLC phosphorylation (Carlile-Klusacek and Rizzo, 2007). Because PAR_1 coupling to $G\alpha_q$ mediates thrombin-induced CCL2 secretion via the RhoA/Rho kinase pathway, we propose that it is likely that the components for secretion in this pathway might integrate on lipid rafts. In contrast, the role of lipid rafts in MAPK signaling remains controversial. For example, $G\alpha_{q/11}$ has been shown to activate p38 MAPK via lipid rafts, whereas two studies have shown that $G\alpha_q$ does not mediate ERK1/2 phosphorylation via such rafts (Hiol *et al.*, 2003; Sugawara *et al.*, 2007). In this study, we report that PAR_1 coupling to $G\alpha_q$ mediates thrombin-induced CCL2 production via ERK1/2 rather than p38 signaling so that current evidence would lead us to propose that the components involved in the transcriptional response may not float together on lipid rafts and would therefore be segregated from the components involved in CCL2 secretion. It is also possible that these pathways involve the assembly of a transduceosome that would act as a relay to assemble and integrate the signals derived from the two pathways in order to optimize the amplitude of downstream signaling. Transduceosomes are most well described in terms of the essential role of the scaffold and anchoring protein, A-kinase anchor proteins (AKAP) in cAMP signaling (Feliciello *et al.*, 2001). cAMP-dependent protein kinase is targeted to discrete subcellular locations by the AKAPs. Localization recruits protein kinase A (PKA) holoenzyme close to its substrate/effector proteins, directing and amplifying the biological effects of cAMP signaling. Although AKAPs were identified on the basis of their interaction with PKA, AKAPs bind other signaling molecules, principally phosphatases and kinases, including notably PKC (Feliciello

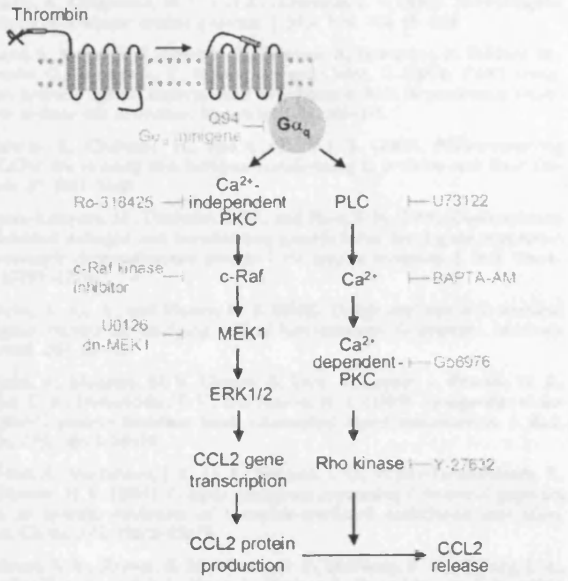

Figure 9. Model for thrombin-induced CCL2 production and release. Thrombin ligation of PAR_1 stimulates $G\alpha_q$. Activation of Ca^{2+}-independent PKC leads to the sequential activation of c-Raf, MEK1 and ERK1/2 to stimulate CCL2 gene transcription. $G\alpha_q$ also leads to the activation of PLC, Ca^{2+}, and Ca^{2+}-dependent PKC to activate Rho kinase (likely via p115RhoGEF, Singh *et al.*, 2007) to mediate CCL2 protein release.

et al., 2001). It is therefore tempting to speculate and propose a role for both lipid rafts and transduceosomes to integrate and assemble the signaling components leading from PAR_1 activation of $G\alpha_q$ to CCL2 gene transcription via the ERK1/2 pathway and CCL2 release via the RhoA/RhoA pathway.

Summary and Therapeutic Implications

We provide compelling evidence that thrombin mediates its potent stimulatory effects on fibroblast CCL2 production and release by coupling to $G\alpha_q$ and the cooperation between ERK1/2 and Rho kinase signaling pathways (Figure 9). Inhibition of the Ca^{2+}-independent PKC-ERK1/2 pathway not only inhibits CCL2 protein levels, but also inhibits intracellular CCL2 protein production and CCL2 mRNA levels, suggesting that this pathway mediates thrombin-induced CCL2 release by influencing CCL2 gene transcription. Inhibition of the PLC-Rho kinase pathway inhibits CCL2 protein release, but has no effect on thrombin-induced intracellular CCL2 protein production and CCL2 mRNA levels. The RhoA/Rho kinases are well known for their regulatory role in stress fiber formation and secretory vesicle trafficking, suggesting that this pathway mediates thrombin-induced CCL2 secretion. Blockade of PAR_1 coupling to $G\alpha_q$ was found to inhibit both ERK1/2 and MLC phosphorylation induced by thrombin, indicating that these two pathways both lie downstream of PAR_1 coupling to $G\alpha_q$.

To the best of our knowledge, this report represents the first demonstration of the cooperation between these two pathways in mediating the stimulatory effects of thrombin, or indeed any other extracellular stimulus, on the induction and release of the potent chemoattractant, CCL2. In this report, we further demonstrate the effectiveness of blocking this response using the recently developed novel PAR_1 antagonist, Q94, which blocks PAR_1 at the intracellular interaction

site with $G\alpha_q$ (Caden Biosciences) and therefore allows more selective targeting of some, but not all, PAR_1-mediated cellular responses. We propose that such an antagonist approach may hold promise for selectively interfering with deleterious thrombin signaling in the context of tissue inflammation and fibroproliferative disease, while preserving other essential PAR_1-mediatedcellular responses.

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REFERENCES

Amano, M., Ito, M., Kimura, K., Fukata, Y., Chihara, K., Nakano, T., Matsuura, Y., and Kaibuchi, K. (1996). Phosphorylation and activation of myosin by Rho-associated kinase (Rho-kinase). J. Biol. Chem. *271*, 20246–20249.

Bachli, E. B., Pech, C. M., Johnson, K. M., Johnson, D. J., Tuddenham, E. G., and McVey, J. H. (2003). Factor Xa and thrombin, but not factor VIIa, elicit specific cellular responses in dermal fibroblasts. J. Thromb. Haemost. *1*, 1935–1944.

Bain, J., McLauchlan, H., Elliott, M., and Cohen, P. (2003). The specificities of protein kinase inhibitors: an update. Biochem. J. *371*, 199–204.

Blumer, K. J., and Johnson, G. L. (1994). Diversity in function and regulation of MAP kinase pathways. Trends Biochem. Sci. *19*, 236–240.

Brandes, R. P., Viedt, C., Nguyen, K., Beer, S., Kreuzer, J., Busse, R., and Gorlach, A. (2001). Thrombin-induced MCP-1 expression involves activation of the p22phox-containing NADPH oxidase in human vascular smooth muscle cells. Thromb. Haemost. *85*, 1104–1110.

Carlile-Klusacek, M., and Rizzo, V. (2007). Endothelial cytoskeletal reorganization in response to PAR1 stimulation is mediated by membrane rafts but not caveolae. Am. J. Physiol. Heart Circ. Physiol. *293*, H366–H375.

Chambers, R. C. (2003). Role of coagulation cascade proteases in lung repair and fibrosis. Eur. Respir. J. Suppl. *44*, 33s–35s.

Cheung, W. M., D'Andrea, M. R., Andrade-Gordon, P., and Damiano, B. P. (1999). Altered vascular injury responses in mice deficient in protease-activated receptor-1. Arterioscler. Thromb. Vasc. Biol. *19*, 3014–3024.

Cobb, M. H., Xu, S., Hepler, J. E., Hutchison, M., Frost, J., and Robbins, D. J. (1994). Regulation of the MAP kinase cascade. Cell. Mol. Biol. Res. *40*, 253–256.

Colotta, F., Sciacca, F. L., Sironi, M., Luini, W., Rabiet, M. J., and Mantovani, A. (1994). Expression of monocyte chemotactic protein-1 by monocytes and endothelial cells exposed to thrombin. Am. J. Pathol. *144*, 975.

Coughlin, S. R. (2000). Thrombin signalling and protease-activated receptors. Nature *407*, 258–264.

Cowley, S., Paterson, H., Kemp, P., and Marshall, C. J. (1994). Activation of MAP kinase kinase is necessary and sufficient for PC12 differentiation and for transformation of NIH 3T3 cells. Cell. *77*, 841–852.

Cunningham, M. A., Rondeau, E., Chen, X., Coughlin, S. R., Holdsworth, S. R., and Tipping, P. G. (2000). Protease-activated receptor 1 mediates thrombin-dependent, cell-mediated renal inflammation in crescentic glomerulonephritis. J. Exp. Med. *191*, 455–462.

Davies, S. P., Reddy, H., Caivano, M., and Cohen, P. (2000). Specificity and mechanism of action of some commonly used protein kinase inhibitors. Biochem. J. *351*, 95–105.

Ellis, C. A., Malik, A. B., Gilchrist, A., Hamm, H., Sandoval, R., Voyno-Yasenetskaya, T., and Tiruppathi, C. (1999). Thrombin induces proteinase-activated receptor-1 gene expression in endothelial cells via activation of Gi-linked Ras/mitogen-activated protein kinase pathway. J. Biol. Chem. *274*, 13718–13727.

Etienne-Manneville, S., and Hall, A. (2002). Rho GTPases in cell biology. Nature *420*, 629–635.

Feliciello, A., Gottesman, M. E., and Avvedimento, E. V. (2001). The biological functions of A-kinase anchor proteins. J. Mol. Biol. *308*, 99–114.

Fiorucci, S., Antonelli, E., Distrutti, E., Severino, B., Fiorentina, R., Baldoni, M., Caliendo, G., Santagada, V., Morelli, A., and Cirino, G. (2004). PAR1 antagonism protects against experimental liver fibrosis. Role of proteinase receptors in stellate cell activation. Hepatology *39*, 365–375.

Fukuhara, S., Chikumi, H., and Gutkind, J. S. (2001). RGS-containing RhoGEFs: the missing link between transforming G proteins and Rho? Oncogene *20*, 1661–1668.

Gharaee-Kermani, M., Denholm, E. M., and Phan, S. H. (1996). Costimulation of fibroblast collagen and transforming growth factor beta1 gene expression by monocyte chemoattractant protein-1 via specific receptors. J. Biol. Chem. *271*, 17779–17784.

Gilchrist, A., Li, A., and Hamm, H. E. (2002). Design and use of C-terminal minigene vectors for studying role of heterotrimeric G proteins. Methods Enzymol. *344*, 58–69.

Gilchrist, A., Mazzoni, M. R., Dineen, B., Dice, A., Linden, J., Proctor, W. R., Lupica, C. R., Dunwiddie, T. V., and Hamm, H. E. (1998). Antagonists of the receptor-G protein interface block Gi-coupled signal transduction. J. Biol. Chem. *273*, 14912–14919.

Gilchrist, A., Vanhauwe, J. F., Li, A., Thomas, T. O., Voyno-Yasenetskaya, T., and Hamm, H. E. (2001). G alpha minigenes expressing C-terminal peptides serve as specific inhibitors of thrombin-mediated endothelial activation. J. Biol. Chem. *276*, 25672–25679.

Goodman, R. B., Strieter, R. M., Martin, D. P., Steinberg, K. P., Milberg, J. A., Maunder, R. J., Kunkel, S. L., Walz, A., Hudson, L. D., and Martin, T. R. (1996). Inflammatory cytokines in patients with persistence of the acute respiratory distress syndrome. Am. J. Respir. Crit. Care Med. *154*, 602–611.

Hamm, H. E. (1998). The many faces of G protein signaling. J. Biol. Chem. *273*, 669–672.

Hamm, H. E., and Gilchrist, A. (1996). Heterotrimeric G proteins. Curr. Opin. Cell Biol. *8*, 189–196.

Hart, M. J., Jiang, X., Kozasa, T., Roscoe, W., Singer, W. D., Gilman, A. G., Sternweis, P. C., and Bollag, G. (1998). Direct stimulation of the guanine nucleotide exchange activity of p115 RhoGEF by Galpha13. Science *280*, 2112–2114.

Hiol, A., Davey, P. C., Osterhout, J. L., Waheed, A. A., Fischer, E. R., Chen, C. K., Milligan, G., Druey, K. M., and Jones, T. L. (2003). Palmitoylation regulates regulators of G-protein signaling (RGS) 16 function. I. Mutation of amino-terminal cysteine residues on RGS16 prevents its targeting to lipid rafts and palmitoylation of an internal cysteine residue. J. Biol. Chem. *278*, 19301–19308.

Hogaboam, C. M., Steinhauser, M. L., Chensue, S. W., and Kunkel, S. L. (1998). Novel roles for chemokines and fibroblasts in interstitial fibrosis. Kidney Int. *54*, 2152–2159.

Hollenberg, M. D., Saifeddine, M., al-Ani, B., and Kawabata, A. (1997). Proteinase-activated receptors: structural requirements for activity, receptor cross-reactivity, and receptor selectivity of receptor-activating peptides. Can. J. Physiol. Pharmacol. *75*, 832–841.

Howell, D. C., Johns, R. H., Lasky, J. A., Shan, B., Scotton, C. J., Laurent, G. J., and Chambers, R. C. (2005). Absence of proteinase-activated receptor-1 signaling affords protection from bleomycin-induced lung inflammation and fibrosis. Am. J. Pathol. *166*, 1353–1365.

Janes, S. M., and Watt, F. M. (2004). Switch from alphavbeta5 to alphavbeta6 integrin expression protects squamous cell carcinomas from anoikis. J. Cell Biol. *166*, 419–431.

Jenkins, R. G., Su, X., Su, G., Scotton, C. J., Camerer, E., Laurent, G. J., Davis, G. E., Chambers, R. C., Matthay, M. A., and Sheppard, D. (2006). Ligation of protease-activated receptor 1 enhances alpha(v)beta6 integrin-dependent TGF-beta activation and promotes acute lung injury. J. Clin. Invest. *116*, 1606–1614.

Kozasa, T., Jiang, X., Hart, M. J., Sternweis, P. M., Singer, W. D., Gilman, A. G., Bollag, G., and Sternweis, P. C. (1998). p115 RhoGEF, a GTPase activating protein for Galpha12 and Galpha13. Science *280*, 2109–2111.

Major, C. D., Santulli, R. J., Derian, C. K., and Andrade-Gordon, P. (2003). Extracellular mediators in atherosclerosis and thrombosis: lessons from thrombin receptor knockout mice. Arterioscler. Thromb. Vasc. Biol. *23*, 931–939.

Marin, V., Farnarier, C., Gres, S., Kaplanski, S., Su, M. S., Dinarello, C. A., and Kaplanski, G. (2001). The p38 mitogen-activated protein kinase pathway plays a critical role in thrombin-induced endothelial chemokine production and leukocyte recruitment. Blood *98*, 667–673.

McLaughlin, J. N., Shen, L., Holinstat, M., Brooks, J. D., Dibenedetto, E., and Hamm, H. E. (2005). Functional selectivity of G protein signaling by agonist peptides and thrombin for the protease-activated receptor-1. J. Biol. Chem. *280*, 25048–25059.

Moore, B. B., Kolodsick, J. E., Thannickal, V. J., Cooke, K., Moore, T. A., Hogaboam, C., Wilke, C. A., and Toews, G. B. (2005). CCR2-mediated recruitment of fibrocytes to the alveolar space after fibrotic injury. Am. J. Pathol. *166*, 675–684.

Phillips, R. J., Burdick, M. D., Hong, K., Lutz, M. A., Murray, L. A., Xue, Y. Y., Belperio, J. A., Keane, M. P., and Strieter, R. M. (2004). Circulating fibrocytes traffic to the lungs in response to CXCL12 and mediate fibrosis. J. Clin. Invest. *114*, 438–446.

Simons, K., and Toomre, D. (2000). Lipid rafts and signal transduction. Nat. Rev. Mol. Cell Biol. *1*, 31–39.

Singh, I., Knezevic, N., Ahmmed, G. U., Kini, V., Malik, A. B., and Mehta, D. (2007). Galphaq-TRPC6-mediated Ca2+ entry induces RhoA activation and resultant endothelial cell shape change in response to thrombin. J. Biol. Chem. *282*, 7833–7843.

Suga, M., Iyonaga, K., Ichiyasu, H., Saita, N., Yamasaki, H., and Ando, M. (1999). Clinical significance of MCP-1 levels in BAl.F and serum in patients with interstitial lung diseases. Eur. Respir. J. *14*, 376–382.

Sugawara, Y., Nishii, H., Takahashi, T., Yamauchi, J., Mizuno, N., Tago, K., and Itoh, H. (2007). The lipid raft proteins flotillins/reggies interact with Galphaq and are involved in Gq-mediated p38 mitogen-activated protein kinase activation through tyrosine kinase. Cell Signal. *19*, 1301–1308.

Suzuki, N., Nakamura, S., Mano, H., and Kozasa, T. (2003). Galpha 12 activates Rho GTPase through tyrosine-phosphorylated leukemia-associated RhoGEF. Proc. Natl. Acad. Sci. USA *100*, 733–738.

Taipale, J., Koli, K., and Keski-Oja, J. (1992). Release of transforming growth factor-beta 1 from the pericellular matrix of cultured fibroblasts and fibrosarcoma cells by plasmin and thrombin. J. Biol. Chem. *267*, 25378–25384.

Tanaka, N., Morita, T., Nezu, A., Tanimura, A., Mizoguchi, I., and Tojyo, Y. (2004). Signaling mechanisms involved in protease-activated receptor-1-mediated interleukin-6 production by human gingival fibroblasts. J. Pharmacol. Exp. Ther. *311*, 778–786.

Trejo, J., Connolly, A. J., and Coughlin, S. R. (1996). The cloned thrombin receptor is necessary and sufficient for activation of mitogen-activated protein kinase and mitogenesis in mouse lung fibroblasts. Loss of responses in fibroblasts from receptor knockout mice. J. Biol. Chem. *271*, 21536–21541.

Vanhauwe, J. F., Thomas, T. O., Minshall, R. D., Tiruppathi, C., Li, A., Gilchrist, A., Yoon, E. J., Malik, A. B., and Hamm, H. E. (2002). Thrombin receptors activate G (o) proteins in endothelial cells to regulate intracellular calcium and cell shape changes. J. Biol. Chem. *277*, 34143–34149.

Vergnolle, N. *et al.* (2004). A role for proteinase-activated receptor-1 in inflammatory bowel diseases. J. Clin. Invest. *114*, 1444–1456.

Vogt, S., Grosse, R., Schultz, G., and Offermanns, S. (2003). Receptor-dependent RhoA activation in G12/G13-deficient cells: genetic evidence for an involvement of Gq/G11. J. Biol. Chem. *278*, 28743–28749.

Vu, T. K., Hung, D. T., Wheaton, V. I., and Coughlin, S. R. (1991). Molecular cloning of a functional thrombin receptor reveals a novel proteolytic mechanism of receptor activation. Cell *64*, 1057–1068.

Walz, D. A., Anderson, G. F., Ciaglowski, R. E., Aiken, M., and Fenton, J. W., 2nd. (1985). Thrombin-elicited contractile responses of aortic smooth muscle. Proc. Soc. Exp. Biol. Med. *180*, 518–526.